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AUTHORITY

US Army Med Research and Mat Cmd, MCMR-RMI-S [70-1y], ltr 6 Jul 2000, Ft Detrick, MD

AD_____

GRANT NUMBER DAMD17-94-J-4057

TITLE: Breast Cancer Resistance to Cyclophosphamide and Other Oxazaphosphorines

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REPORT DATE: October 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Annual (1 Oct 95 - 30 Sep 96)

4. TITLE AND SUBTITLE
Breast Cancer Resistance to Cytophosphamide and Other Oxazaphosphorines

5. FUNDING NUMBERS
DAMD17-94-J-4057

6. AUTHOR(S)
Norman E. Sladek, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Minnesota
Minneapolis, Minnesota 55415-1226

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Cyclophosphamide is widely used in the treatment of metastatic breast cancer. Unfortunately, emergence of drug-resistant tumor cell populations limits its usefulness somewhat. Class 3 and class 1 aldehyde dehydrogenases (ALDH-3 and ALDH-1, respectively) have been shown to catalyze the detoxification of cyclophosphamide and other oxazaphosphorines. Predictably, then, relatively elevated levels of ALDH-3 and ALDH-1 have been shown to account for resistance to these agents in several cultured breast and other tumor cell models. It follows that cellular resistance to these agents on the part of clinical breast cancers could be due to overexpression of ALDH-3 and/or ALDH-1. Our finding that ALDH-3 and ALDH-1 levels vary widely in primary and metastatic breast tumor tissues supports this notion. Xenobiotics that are abundantly present in the diet/environment, e.g., methylcholanthrene and catechol, rapidly, coordinately and reversibly induce ALDH-3 in cultured breast cancer cell models, thus, rapidly effecting reversible resistance to cyclophosphamide. Induction by methylcholantherene and other Ah receptor ligands may be possible only in estrogen receptor-positive cells whereas that by catechol and other phenolic antioxidants is estrogen receptor-independent. In any case, the environment and diet may influence the therapeutic efficacy of cyclophosphamide. Three relatively specific inhibitors of tumor cell ALDH-3, viz., gossypol and two chlorpropamide analogues, have been identified. Each of these inhibitors sensitized cultured human breast adenosarcoma MCF-7 cells, otherwise insensitive to the oxazaphosphorines because they express relatively large amounts of ALDH-3, to these agents, thereby establishing the therapeutic potential of combining these inhibitors with an oxazaphosphorine when breast cancer cell insensitivity to the latter is due to significantly high cellular levels of ALDH-3. Especially attractive in that regard is that gossypol itself is toxic to breast cancer cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Cyclophosphamide, Reversal of Drug Resistance, Aldehyde Dehydrogenase, Chemotherapy, Anatomical Samples, Mafosfamide, 4-hydroperoxycyclophosphamide, Breast Cancer	126
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

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M. E. Sládek 10.21.96

PI - Signature Date

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INTRODUCTION

Cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide are antineoplastic agents collectively referred to as oxazaphosphorines [Sladek, 1994]. Each of these is a prodrug, i.e., per se, without cytotoxic activity. Salient features of the metabolic activation of oxazaphosphorines are presented in Figure 1. Oxazaphosphorines are clinically effective; they play a lead role in the treatment of breast cancer until resistant subpopulations become the dominant population. An understanding of how resistance to these agents is effected would likely to be of value because measures may then become apparent as to how to reverse, and/or prevent, it. It is this understanding which is the overall objective of our first-generation investigations.

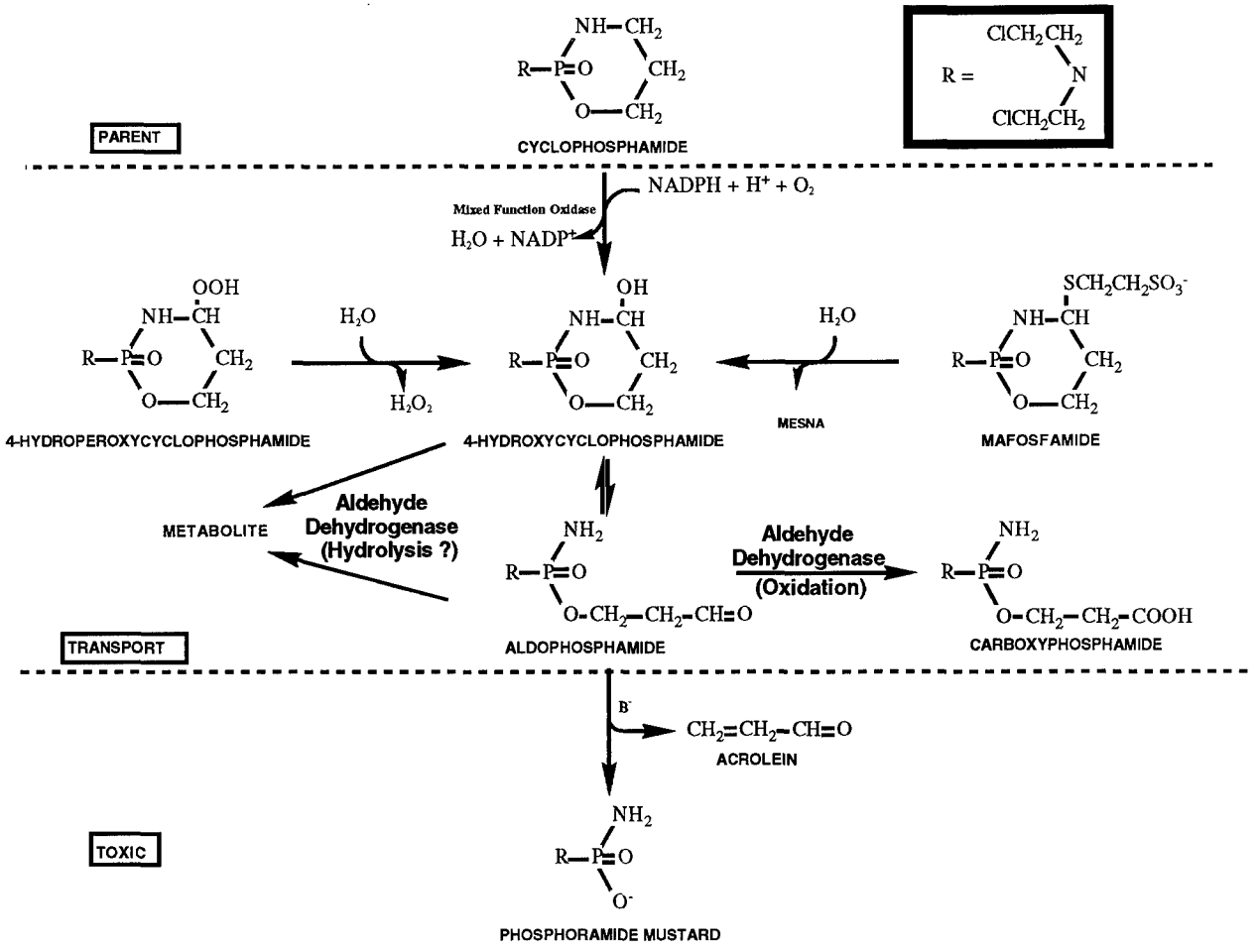


Figure 1. Salient features of oxazaphosphorine metabolism. The prodrugs, cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide, each give rise to 4-hydroxycyclophosphamide which exists in equilibrium with its ring-opened tautomer, aldophosphamide. 4-Hydroxycyclophosphamide and aldophosphamide are, themselves, also without cytotoxic activity. However, aldophosphamide gives rise to acrolein and phosphoramide mustard, each of which is cytotoxic; the latter effects the bulk of the therapeutic action effected by the oxazaphosphorines [Sladek, 1994]. Alternatively, aldophosphamide can be further oxidized to carboxyphosphamide by certain aldehyde dehydrogenases [Manthey et al., 1990; Dockham et al., 1992; Sreerama and Sladek, 1993a, 1994; Sladek, 1994]. Carboxyphosphamide is without cytotoxic activity nor does it give rise to a cytotoxic metabolite. Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide is, therefore, properly viewed as an enzyme-catalyzed detoxification of the oxazaphosphorines. Aldehyde dehydrogenase-catalyzed hydrolysis of 4-hydroxycyclophosphamide and/or aldophosphamide to an inactive metabolite is also shown but is only a possibility, i.e., it is yet to be demonstrated.

Most pertinent to these investigations is the irreversible detoxification that occurs when NAD(P)-dependent aldehyde dehydrogenases catalyze the oxidation of a pivotal metabolite, viz., aldophosphamide, to carboxyphosphamide, Figure 1. Human class-1, -2 and -3 aldehyde dehydrogenases, viz., ALDH-1, ALDH-2 and ALDH-3, respectively, as well as succinic semialdehyde dehydrogenase, all catalyze the oxidation of aldophosphamide to carboxyphosphamide, but not equally well [Dockham et al., 1992; Sladek, 1993, 1994; Sreerama and Sladek, 1993a, 1994].

Aldehyde dehydrogenases are bifunctional enzymes in that they catalyze not only the oxidation of aldehydes, but also the hydrolysis of ester bonds. Several such bonds are present in 4-hydroxycyclophosphamide and aldophosphamide, Figure 2. Whether aldehyde dehydrogenases catalyze the hydrolysis of either of these intermediates to an irreversibly inactive metabolite is not known.

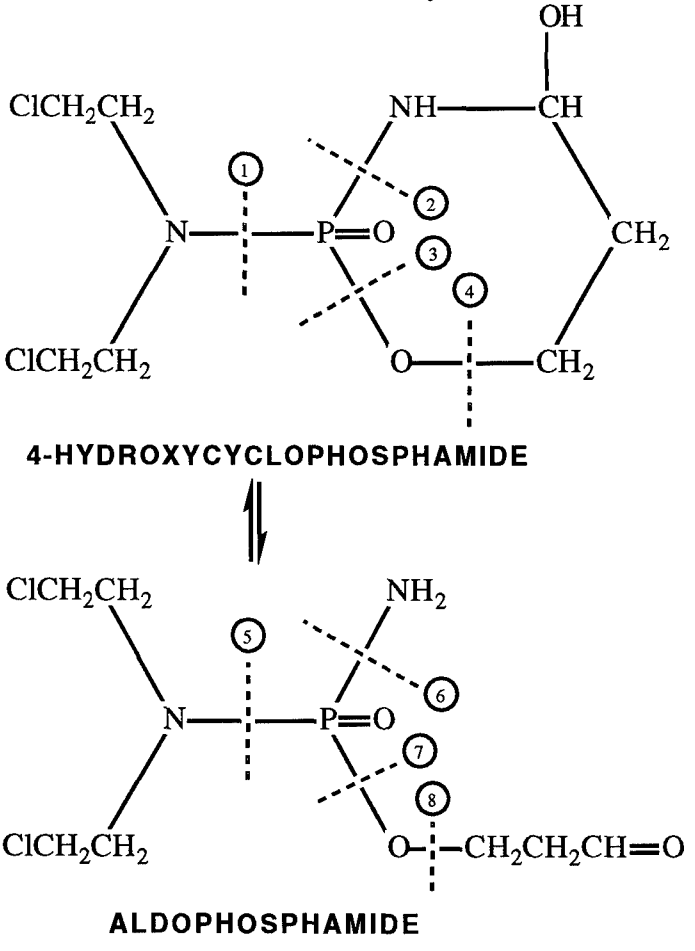


Figure 2. Aldehyde dehydrogenase-catalyzed hydrolysis of 4-hydroxycyclophosphamide and aldophosphamide: possibilities. Hydrolysis of 4-hydroxycyclophosphamide at (1) and aldophosphamide at (5) would give rise to bis-(2-chloroethyl)-amine. Thus, hydrolysis at (1) or (5) cannot account for oxazaphosphorine detoxification, because bis-(2-chloroethyl)-amine is more cytotoxic than are the prodrugs, e.g., mafosfamide and 4-hydroperoxycyclophosphamide, from whence it would originate [Sreerama and Sladek, 1993a; Sladek, 1994]. Hydrolysis of aldophosphamide at (7) and (8) would give rise to phosphoramide mustard. Thus, hydrolysis at (7) and (8) cannot account for the oxazaphosphorine detoxification, because phosphoramide mustard is more cytotoxic than are the prodrugs, e.g., mafosfamide, 4-hydroperoxycyclophosphamide, from whence it would originate [Sreerama and Sladek, 1993a, 1994; Sladek, 1994].

Using cultured human breast adenocarcinoma MCF-7/0 cells and two oxazaphosphorine-resistant sublines derived therefrom, viz., MCF-7/OAP (stable resistance achieved by growing the parent MCF-7/0

cells in the presence of continuously increasing concentrations of 4-hydroperoxycyclophosphamide for many months [Frei et al., 1988]) and MCF-7/PAH (transient resistance achieved by growing the parent MCF-7/0 cells in the presence of a polycyclic aromatic hydrocarbon (PAH), e.g., 3 μM 3-methylcholanthrene, for 5 days [Sreerama and Sladek, 1993b, 1994]), we have demonstrated that ALDH-3 is an important determinant of cellular sensitivity to the oxazaphosphorines [Sreerama and Sladek 1993a,b, 1994]. MCF-7 cells do not contain any of the mixed function oxidases that activate cyclophosphamide, Figure 1. Thus, we used mafosfamide and/or 4-hydroperoxycyclophosphamide rather than cyclophosphamide in all of these and other experiments with cultured MCF-7 cells because these agents, like cyclophosphamide, give rise to 4-hydroxycyclophosphamide, but they do so in the absence of any enzyme involvement, Figure 1.

The above investigations led us to hypothesize that 1) clinical breast cancer cellular resistance to cyclophosphamide and other oxazaphosphorines is the consequence of elevated ALDH-3 levels, 2) ALDH-3 mediates cellular resistance to oxazaphosphorines by catalyzing the oxidative and/or hydrolytic detoxification of these agents, 3) inhibitors of the detoxifying reaction can be identified and utilized to reverse the resistance, 4) hypomethylation of ALDH-3 genomic DNA accounts for oxazaphosphorine- and activated Ah receptor-induced ALDH-3 overexpression, 5) activated Ah receptor-induced ALDH-3 overexpression can only occur in cells that are estrogen receptor-positive, and 6) agents known to induce xenobiotic-metabolizing enzymes via the antioxidant responsive element (ARE) will also induce ALDH-3 overexpression, since the ARE consensus sequence is present in the 5'-flanking region of the ALDH-3 gene. Testing of these hypotheses was divided into seven tasks (statement of work), viz., 1) quantify cellular ALDH-3 levels in surgically removed human breast tumor samples, 2) ascertain the ability of ALDH-3s to catalyze the oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide) at a rate sufficient to account for the oxazaphosphorine-specific acquired resistance exhibited in our model systems, 3) synthesize and identify agents that inhibit the ALDH-3-catalyzed oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide), 4) evaluate identified inhibitors of the relevant ALDH-3 activity with respect to their ability to sensitize our oxazaphosphorine-resistant models to the oxazaphosphorines, 5) identify the molecular basis for the apparent overexpression of ALDH-3s in our model systems, 6) ascertain the ability of Ah receptor ligands to induce ALDH-3 overexpression and oxazaphosphorine-specific acquired resistance in estrogen receptor-positive and -negative breast cancer cell lines that lack and express Ah receptors, and 7) ascertain the ability of ligands for ARE to induce ALDH-3 activity and oxazaphosphorine-specific acquired resistance in our model system.

Repository breast tumor samples and culture models, viz., MCF-7/0, MCF-7/OAP and MCF-7/PAH, were chosen to test the hypotheses delineated above. Methods/technology to be used in testing the above-listed hypotheses include immunocytochemistry, ELISA, ultracentrifugation, density-gradient centrifugation, column and thin-layer chromatography, HPLC, spectrophotometry to monitor catalytic

rates, synthetic organic chemistry, cell culture and colony-forming assays, RT-PCR, Northern and Southern blot analysis, methylation-sensitive restriction enzyme diagnosis and receptor binding assays.

Results of investigations conducted in months 13 through 24 are summarized in the text that follows. They will support the following:

Original Tasks	Months	Progress
1	1 - 48	Nearly completed
2	1 - 24	Nearly completed
3	1 - 24	Partially completed
4	6 - 30	Partially completed
5	24 - 48	Not yet addressed
6	12 - 42	Partially completed
7	36 - 48	Completed

BODY

Task # 1: Quantify cellular ALDH-3 levels in surgically removed human breast tumor samples.

Cellular levels of ALDH-3 in 171 surgically removed primary (122) and metastatic (49) human breast tumor samples were semiquantified with the aid of immunocytochemical staining methodology. ALDH-1 is another known determinant of cellular sensitivity to the oxazaphosphorines [reviewed in Sladek, 1993]. Thus, ALDH-1 levels in breast tumor tissues, together with those of ALDH-3, are more likely to correlate with clinical outcome than are ALDH-3 levels alone. Hence, semiquantification of ALDH-1 levels was added to the original task. Results of these investigations are summarized in Tables 1 (ALDH-3) and 2 (ALDH-1).

Table 1. Immunocytochemical semiquantification of ALDH-3 levels in human primary and metastatic breast tumor samples.

Breast Cancer	# Sample	ALDH-3[a]			
		0	1	2	3
Primary	122	49 (40%)	46 (38%)	24 (20%)	3 (2%)
Metastatic	49	6 (12%)	24 (49%)	17 (35%)	2 (4%)

[a]Staining intensities were rated on a 0 to 3 scale: no visible staining was scored as 0, borderline, faint staining was scored as 1, and clearly visible, progressively intense, staining was scored as 2 and 3.

The immunocytochemical staining methodology utilized to visualize and semiquantify ALDH-3 and ALDH-1 present in breast tumor tissue was developed and standardized with the aid of frozen human liver and stomach mucosa samples, and several cultured cell lines, that contained known amounts of ALDH-3 and/or ALDH-1 activities. The optimized method proved to be highly sensitive and reproducible when used on frozen breast tumor tissue sections which usually contained significantly lower amounts of aldehyde dehydrogenase as compared to that contained by human liver and stomach mucosa. Briefly, repository frozen tissue sections were first incubated with polyclonal antibodies specific for ALDH-3 or ALDH-1 and then with a biotin-linked secondary antibody, after which they were incubated with an avidin-biotin complex conjugated to horse radish peroxidase and then with diaminobenzidine and H_2O_2 for color development. Patient and tumor characteristics, treatments, and treatment outcomes were obtained from the archived medical record that had been generated for each subject.

Table 2. Immunocytochemical semiquantification of ALDH-1 levels in human primary and metastatic breast tumor samples.

Breast Cancer	# Samples	ALDH-1[a]			
		0	1	2	3
Primary	122	21 (17%)	41 (34%)	50 (41%)	10 (8%)
Metastatic	49	4 (8%)	20 (41%)	20 (41%)	5 (10%)

[a]Staining intensities were rated on a 0 to 3 scale: no visible staining was scored as 0, borderline, faint staining was scored as 1, and clearly visible, progressively intense, staining was scored as 2 and 3.

Average ALDH-3 and ALDH-1 values were 0.84 and 1.40, respectively, in the primary breast cancer samples. They were 1.31 and 1.53, respectively, in the metastatic breast cancer samples. There was no correlation between ALDH-3 and ALDH-1 levels, nor between either enzyme level and the patient's age or estrogen or progesterone receptor status.

Most of the primary tumor samples were obtained from patients who had not been, nor were they going to be at the time that samples were taken, treated with chemotherapeutic agents. On the other hand, most of the metastatic tumors were obtained from patients who had been, and/or were going to be, treated with chemotherapeutic agents, most commonly, cyclophosphamide, adriamycin, methotrexate, 5-fluorouracil and/or vincristine. Usually, these agents were given in one of several combinations.

Average ALDH-3 and ALDH-1 levels were slightly higher (approximately + 0.2 and + 0.4 units, respectively) in metastatic tumor cells that survived exposure to cyclophosphamide alone or to cyclophosphamide and methotrexate, 5-fluorouracil and/or vincristine than were those in metastatic tumor cells that had not been exposed to these drugs. Enzyme levels were not altered by the inclusion of adriamycin, tamoxifen, or radiation in the therapeutic regimen.

Average ALDH-1 levels were higher (+ 0.9 units) in metastatic tumors that, upon subsequent treatment, "did not respond" (tumor size did not decrease or even increased) to chemotherapeutic regimens that included cyclophosphamide than were those in metastatic tumors that, upon subsequent treatment, "did respond" (tumor size decreased) to these chemotherapeutic regimens. Average ALDH-1 levels were very slightly, and not significantly, lower (- 0.13 units) in metastatic tumors that, upon subsequent treatment, "did not respond" to chemotherapeutic regimens that did not include cyclophosphamide than were those in metastatic tumors that, upon subsequent treatment, "did respond" to these chemotherapeutic regimens.

Average ALDH-3 levels were higher (+ 0.3 units) in metastatic tumors that, upon subsequent treatment, "did not respond" to chemotherapeutic regimens that included cyclophosphamide than were those in metastatic tumors that, upon subsequent treatment, "did respond" to these chemotherapeutic regimens. However, average ALDH-3 levels were also higher (+ 0.3 units) in metastatic tumors that, upon subsequent treatment, "did not respond" to chemotherapeutic regimens that did not include cyclophosphamide than were those in metastatic tumors that, upon subsequent treatment, "did respond" to these chemotherapeutic regimens.

We also quantified ALDH-3 and ALDH-1, as well as global glutathione S-transferase, glutathione S-transferase α, μ, π and DT-diaphorase, levels in normal (26) and malignant (112) breast tissue samples procured from the Cooperative Human Tissue Network, Midwestern Division, Columbus, Ohio, Figures 3 and 4 and Table 3. Catalytic assays (spectrophotometric) and enzyme-linked immunosorbent assays (ELISAs) were used for this purpose. Global glutathione S-transferase, glutathione S-transferase α, μ, π and DT-diaphorase levels varied widely, and, as in the investigations reported above, ALDH-3 and ALDH-1, each also varied widely. Coordinated elevation of ALDH-3, global glutathione S-transferase and DT-diaphorase levels was only occasionally observed.

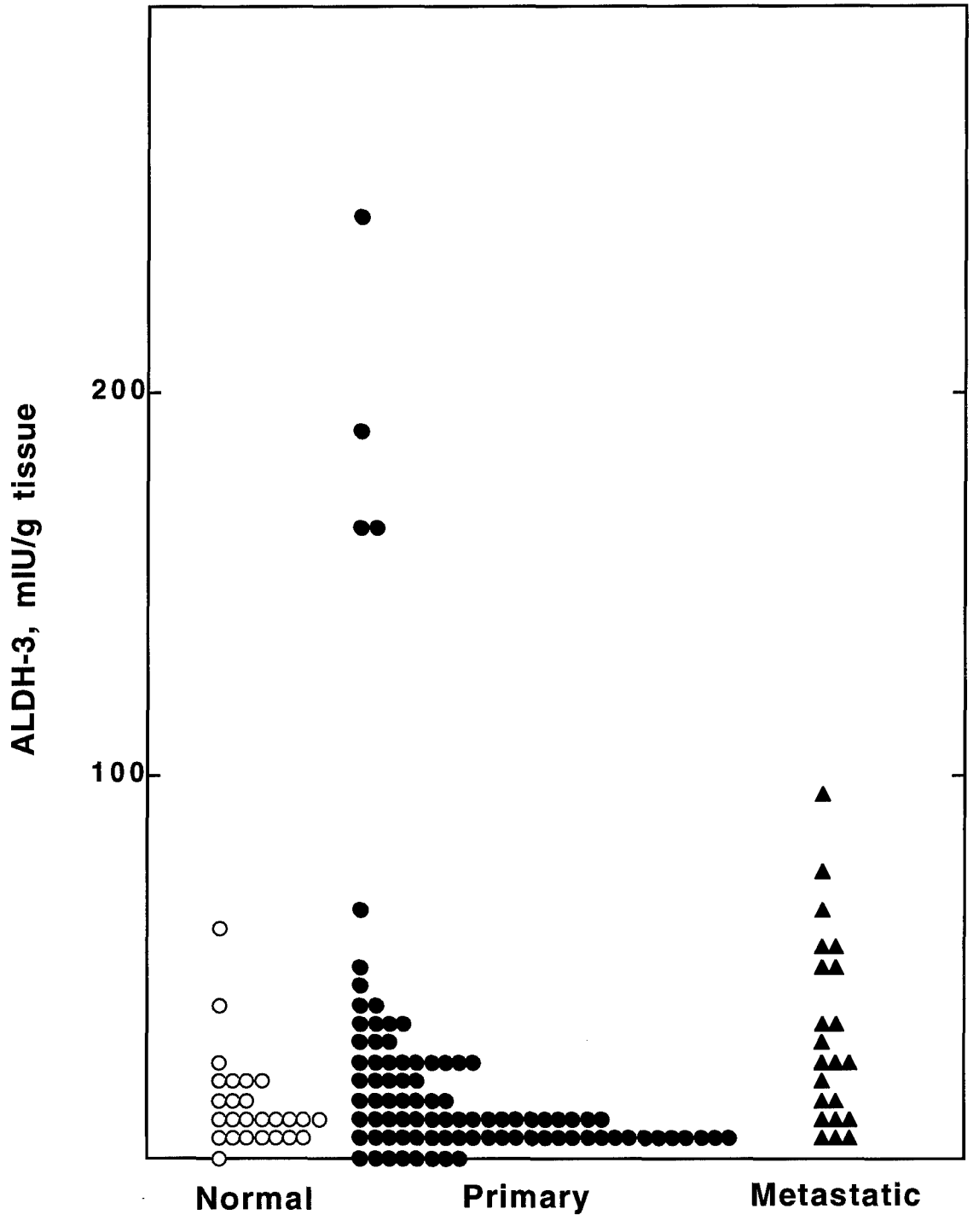


Figure 3. ALDH-3 levels in human normal breast (n = 26), and primary (n = 90) and metastatic (n = 22) breast tumor, tissue samples. Tumor samples were obtained from the Cooperative Human Tissue Network, Midwestern Division, Columbus, Ohio. Whole homogenates and 105,000 g (1 hr) supernatant fractions obtained therefrom were prepared, and ALDH-3 activity was quantified, as described previously [Sreerama and Sladek, 1993a, 1994]; benzaldehyde (4 mM) and NADP (4 mM) were used as substrate and cofactor, respectively. Points are means, rounded off for clarity of presentation to zero if they were < 2.5 mIU/g and to 5 mIU/g or the nearest multiple thereof if they were ≥ 2.5 mIU/g, of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 112 patients.

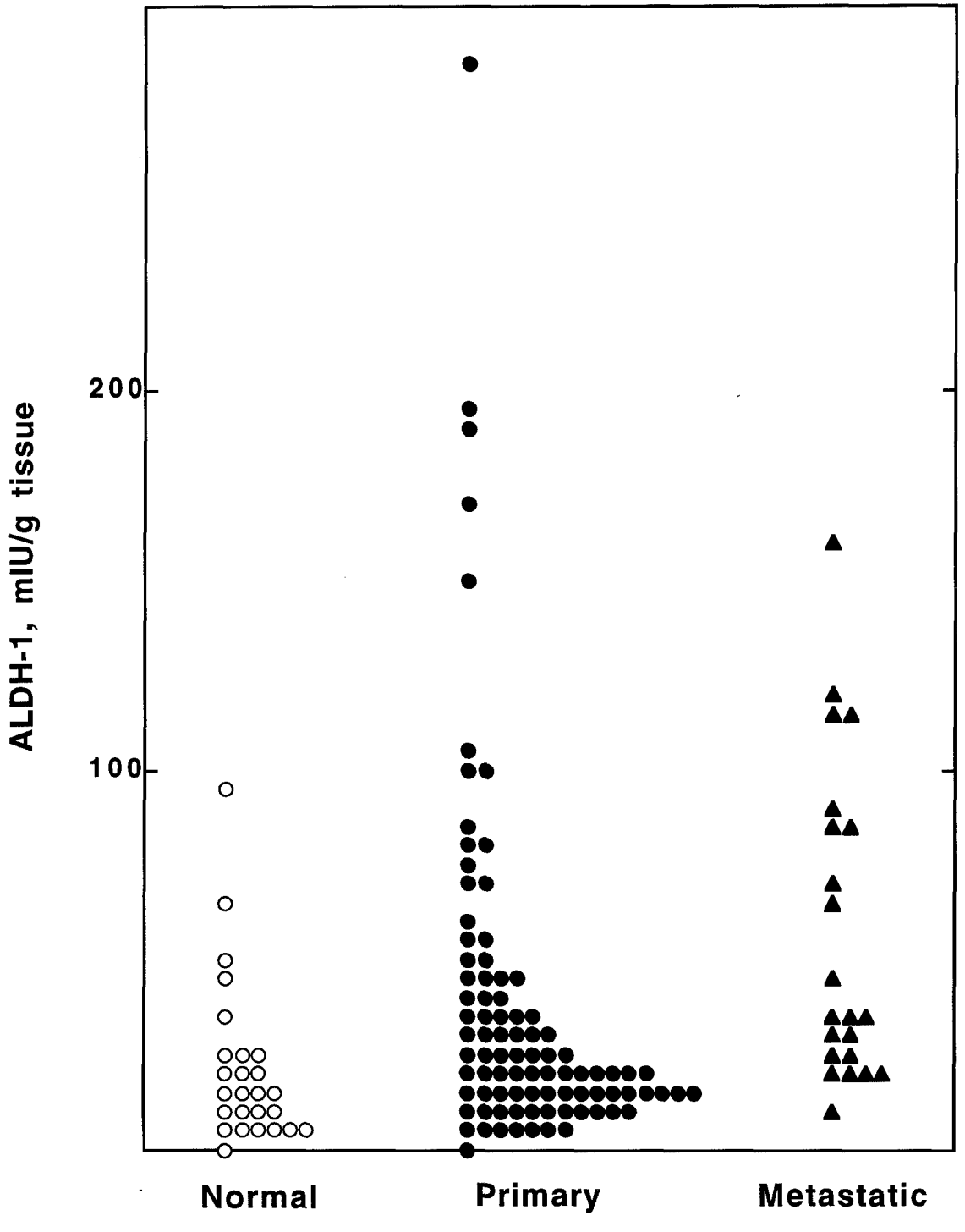


Figure 4. ALDH-1 levels in human normal breast (n = 26), and primary (n = 90) and metastatic (n = 22) breast tumor, tissue samples. Tumor samples were obtained from the Cooperative Human Tissue Network, Midwestern Division, Columbus, Ohio. Whole homogenates and 105,000 g (1 hr) supernatant fractions obtained therefrom were prepared and ALDH-1 activity was quantified, as described previously [Sreerama and Sladek, 1993a, 1994]; acetaldehyde (4 mM) and NAD (4 mM) were used as substrate and cofactor, respectively. Points are means, rounded off for clarity of presentation to zero if they were < 2.5 mIU/g and to 5 mIU/g or the nearest multiple thereof if they were ≥ 2.5 mIU/g, of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 112 patients.

Table 3. ALDH-1, ALDH-3, DT-diaphorase and glutathione S-transferase levels in human normal breast (n = 26), and primary (n = 90) and metastatic (n = 22) breast tumor, tissue samples[a]

Enzyme	mIU/g Breast Tissue					
	Normal		Primary		Metastatic	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
ALDH-3	14 ± 12	2 - 59	22 ± 40	1 - 247	33 ± 25	5 - 93
ALDH-1	21 ± 22	2 - 97	38 ± 47	1 - 287	57 ± 42	9 - 159
DT-Diaphorase	438 ± 690	23 - 2700	1190 ± 1520	10 - 6250	1110 ± 810	84 - 2720
Global GST	920 ± 1050	56 - 4550	2400 ± 2030	123 - 8880	3330 ± 2380	464 - 8130
GST-α[b]	125 ± 171	0 - 900	293 ± 427	0 - 2500	501 ± 605	0 - 1700
GST-μ[c]	96 ± 129	0 - 522	301 ± 560	0 - 3400	295 ± 654	0 - 3050
GST-π	694 ± 829	100 - 3800	1730 ± 1410	120 - 6200	2360 ± 1800	400 - 6500

[a]Preparation of soluble (105,000 x g supernatant) fractions from human normal breast tissues (n = 26) and breast tumor tissues (n = 112), and spectrophotometric quantification of ALDH-1, ALDH-3, DT-diaphorase, and global glutathione S-transferase (GST) levels were as described in Sreerama and Sladek [1993a, 1994]. Acetaldehyde and NAD, 4 mM each, were the substrate and cofactor, respectively, for ALDH-1. Benzaldehyde and NADP, 4 mM each were the substrate and cofactor, respectively, for ALDH-3. 1-Chloro-2,4-dinitrobenzene and glutathione, 1 mM each, were the substrate and cofactor respectively, for global GST. Substrate, cofactor and inhibitor for DT-diaphorase were, respectively, 2,6-dichlorophenol-indophenol (40 μM), NAD(P)H (160 μM) and dicumarol (10 μM). Quantification of GST α, μ and π levels in soluble (105,000 g supernatant) fractions was by an enzyme-linked immunosorbent assay (ELISA) as described by Hornbeck et al. [1991].

[b]GST-α values were zero in 4 of 26 (15%) normal breast tissue samples, 14 of 90 (16%) primary breast tumor samples, and 5 of 22 (23%) metastatic breast tumors samples. Zero values were included in the calculation of mean values.

[c]GST-μ values were zero in 9 of 26 (35%) normal breast tissue samples, 38 of 90 (42%) primary breast tumor samples, and 11 of 22 (50%) metastatic breast tumors samples. Zero values were included in the calculation of mean values.

Task # 2: Ascertain the ability of class 3 aldehyde dehydrogenases to catalyze the oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide) at a rate sufficient to account for the oxazaphosphorine-specific acquired resistance exhibited in our model systems.

We have previously demonstrated that, as judged by the formation of NAD, cytosolic class 3 aldehyde dehydrogenases purified from MCF-7/OAP and MCF-7/PAH cells catalyze the oxidation of aldophosphamide to carboxyphosphamide, and, moreover, that cytosolic fractions prepared from these cells, predictably, catalyze this reaction as well, albeit seemingly not very rapidly, viz., 0.28 and 0.2 μmol/min/10^9 cells, respectively [Sreerama and Sladek, 1993a, 1994]. In the present investigation, we ascertained that, as judged by the formation of carboxyphosphamide, purified tALDH-3, as well as cytosolic fractions prepared from MCF-7/0/CAT cells (MCF-7/0 cells grown in the presence of a phenolic antioxidant, e.g., catechol (CAT) for five days to transiently induce ALDH-3 and the associated

oxazaphosphorine-specific resistance [Sreerama et al., 1995a]), catalyze the oxidation of aldophosphamide, Figure 5. No unidentified NBP-positive metabolites were present. Amounts of 4-hydroxycyclophosphamide/aldophosphamide and carboxyphosphamide present in MCF-7/0/CAT cells after 30 min exposure to mafosfamide were approximately equal. Thus it would appear that although the reaction rate is seemingly slow, ALDH-3-mediated oxazaphosphorine-specific resistance results solely from ALDH-3-catalyzed oxidation of aldophosphamide to carboxyphosphamide, the latter being without cytotoxic activity.

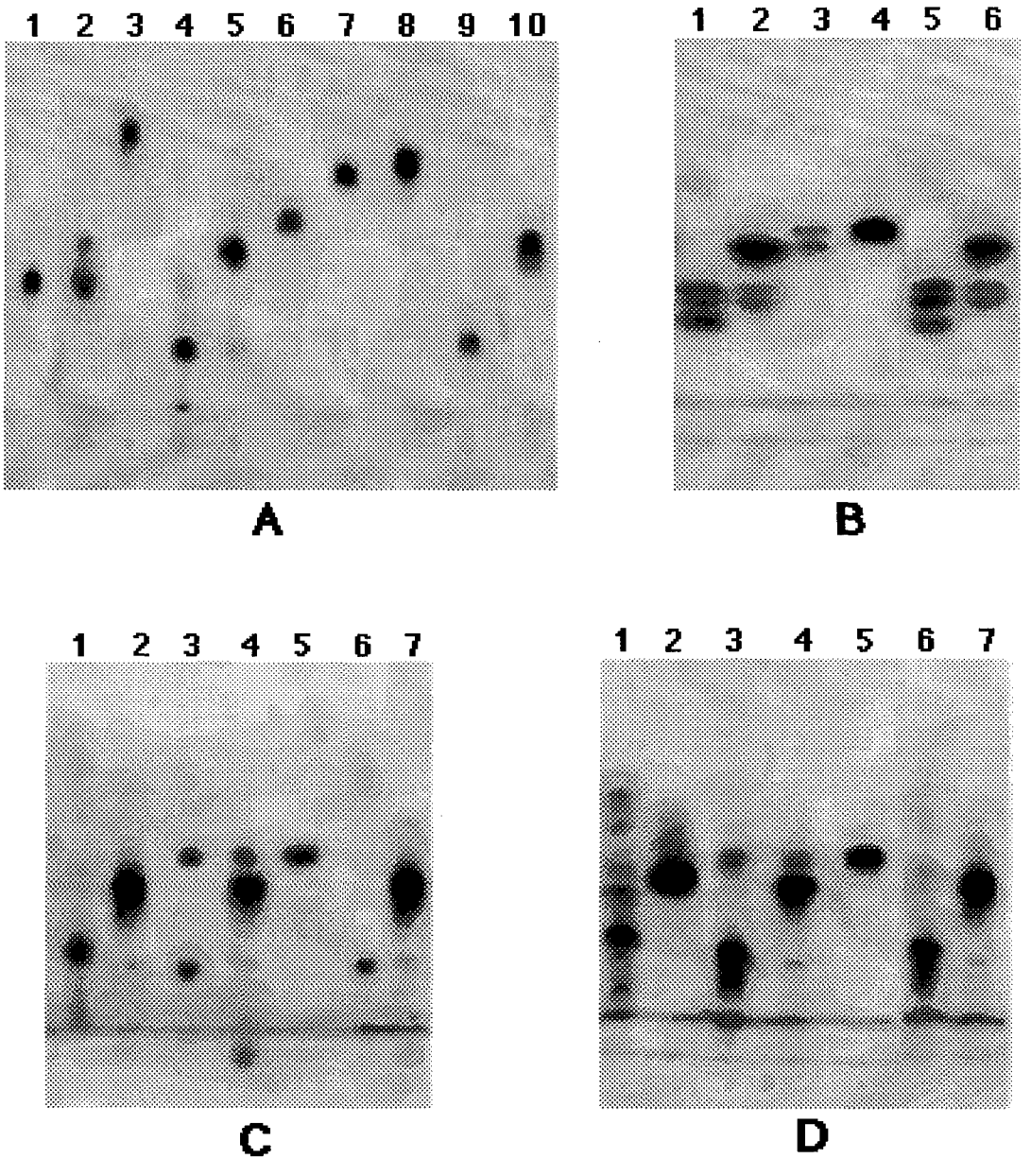
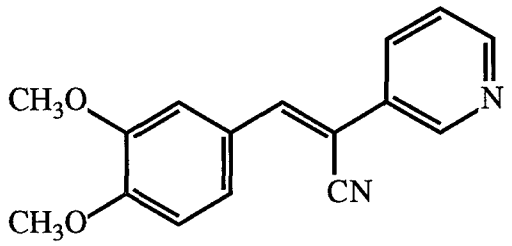


Figure 5. Oxazaphosphorine metabolism catalyzed by purified rALDH-1 and tALDH-3, and intact MCF-7/0/CAT cells: thin-layer chromatographic analysis. Purification of tALDH-3, and preparation of cytosolic fractions, from MCF-7/0/CAT cells was as before [Sreerama and Sladek, 1993a, 1994]. Generation and purification of human recombinant ALDH-1 (rALDH-1) was as described in last year's progress report and by Devaraj et al. [1997]. Incubation of purified tALDH-3 or rALDH-1 with 4-hydroxycyclophosphamide/aldophosphamide was for 15 min, and exposure of 3 x 10^7 MCF-7/0/CAT cells to mafosfamide was for 30 min, as described previously [Dockham et al, 1992; Sreerama and Sladek, 1993a; Sreerama et al.,

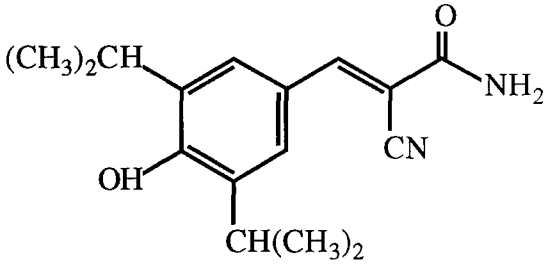
1995a]. A thin-layer chromatography/NBP/computer-assisted scanning/image analysis assay was used to separate and then quantify 4-hydroperoxycyclophosphamide, mafosfamide or 4-hydroxycyclophosphamide/aldophosphamide and their respective metabolites. Semicarbazide was added, when called for, to the incubation mixture at the end of the initial 15 min incubation period with purified enzymes, to a suspension of sonicated (4°C, 10 sec) MCF-7/0/CAT cells that had been exposed to 1 mM mafosfamide for 30 min and then separated from the culture medium by centrifugation (9,000 g, 4°C, 2 min) through a silicone oil/mineral oil (4:1) layer, and to the cell-free aqueous layer (incubation media) remaining on top of the oil layer after centrifugation, and incubation was continued for 5 min to trap 4-hydroxycyclophosphamide/aldophosphamide(hydrate) as the semicarbazone. Sonicated MCF-7/0/CAT cells were then centrifuged at 105,000 g and 4°C for 60 min to obtain 105,000 g soluble fractions. Aliquots of this and the other preparations were then separated on HPTLC high performance LHP-K silica gel plates (Whatman Inc., Clifton, NJ) that had been pre-eluted twice with methanol and dried at 140°C each time for 30 min. TLC was carried out in glass TLC tanks. The mobile phase was butanol:water (20:3). Following separation, the plates were dried with cold air, sprayed with a 5% solution of 4-(p-nitrobenzyl)pyridine (NBP) in acetone/0.2M sodium acetate buffer pH 4.6 (8:2 v/v), baked in a hot air oven at 140°C for 10 min, cooled by blowing cold air over them, and dipped in a 3% methanolic KOH solution to visualize (brilliant violet-blue spots) the compounds of interest [Sladek, 1973]. Because the color is stable for only a few minutes, the developed, still moist, plate was immediately transferred into a polyethylene bag which was then sealed, and scanned with the aid of a Color OneScanner connected to a Power Macintosh computer equipped with "Ofoto 2.0" scanning software (Apple Computer, Inc., Cupertino, CA). Image analysis and quantification of 4-hydroperoxycyclophosphamide, mafosfamide and their metabolites on the scanned image was performed with the aid of Image 1.6 software (NIH, Bethesda, MD) in a Power Macintosh with reference to authentic standards. Plate **A**: Authentic cyclophosphamide analogues and metabolites. Lane 1, mafosfamide; Lane 2, mafosfamide incubated with semicarbazide; Lane 3, 4-hydroperoxycyclophosphamide; Lane 4, 4-hydroxycyclophosphamide/aldophosphamide; Lane 5, 4-hydroxycyclophosphamide/aldophosphamide incubated with semicarbazide; Lane 6, carboxyphosphamide; Lane 7, 4-ketocyclophosphamide; Lane 8, alcophosphamide; Lane 9; phosphoramide mustard; and Lane 10, nornitrogen mustard. Plate **B**: MCF-7/0/CAT cells incubated with mafosfamide for 30 min. Lane 1, cell-free aqueous layer; Lane 2 cell-free aqueous layer incubated with semicarbazide; Lane 3, MCF-7/0/CAT cell-lysate incubated with semicarbazide; Lane 4, authentic carboxyphosphamide; Lane 5, complete incubation medium except for MCF-7/0/CAT cells (blank reaction); and lane 6, complete incubation medium except for MCF-7/0/CAT cells incubated with semicarbazide. Plate **C**: rALDH-1 incubated with 4-hydroxycyclophosphamide/aldophosphamide for 15 min. Lane 1, 4-hydroxycyclophosphamide/aldophosphamide; Lane 2, 4-hydroxycyclophosphamide/aldophosphamide incubated with semicarbazide; Lane 3, rALDH-1 incubated with 4-hydroxycyclophosphamide/aldophosphamide; Lane 4, rALDH-1 incubated with 4-hydroxycyclophosphamide/aldophosphamide and then with semicarbazide; Lane 5, authentic carboxyphosphamide; Lane 6, complete reaction mixture except for rALDH-1 (blank reaction); and Lane 7, complete reaction mixture except for rALDH-1 incubated with semicarbazide. Plate **D**: tALDH-3 incubated with 4-hydroxycyclophosphamide/aldophosphamide for 15 min. Lane 1, 4-hydroxycyclophosphamide/aldophosphamide; Lane 2, 4-hydroxycyclophosphamide/aldophosphamide incubated with semicarbazide; Lane 3, tALDH-3 incubated with 4-hydroxycyclophosphamide/aldophosphamide; Lane 4, tALDH-3 incubated with 4-hydroxycyclophosphamide/aldophosphamide and then with semicarbazide; Lane 5, authentic carboxyphosphamide; Lane 6, complete reaction mixture except for tALDH-3 (blank reaction); and Lane 7, complete reaction mixture except for tALDH-3 incubated with semicarbazide.

Task # 3: Synthesize and identify agents that inhibit the ALDH-3-catalyzed oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide).

Others [Poole et al., 1993] have found that a wide variety of cinnamic acid derivatives inhibited "rat low Km mitochondrial aldehyde dehydrogenase" (ALDH-2) but not lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase or a "rat high Km mitochondrial aldehyde dehydrogenase." Inhibition was competitive with respect to the cofactor, viz., NAD. The sensitivity of class 3 aldehyde dehydrogenases to these agents was not tested. We were able to procure four of these agents, Figure 6, from Dr. A. T. Hudson of The Wellcome Foundation, Beckenham, Kent, England and proceeded to do so.



567C91



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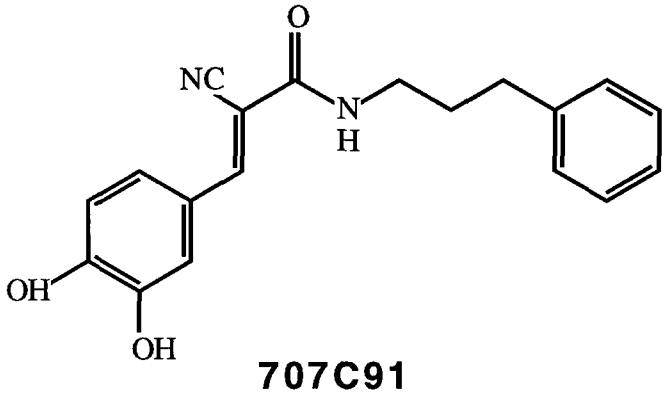
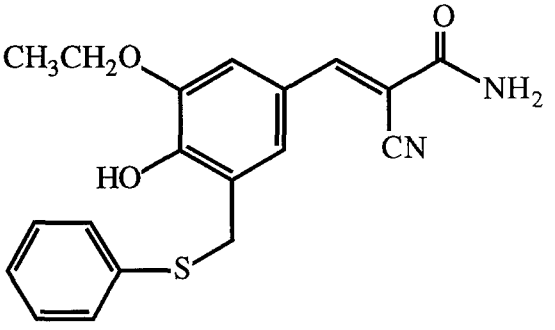
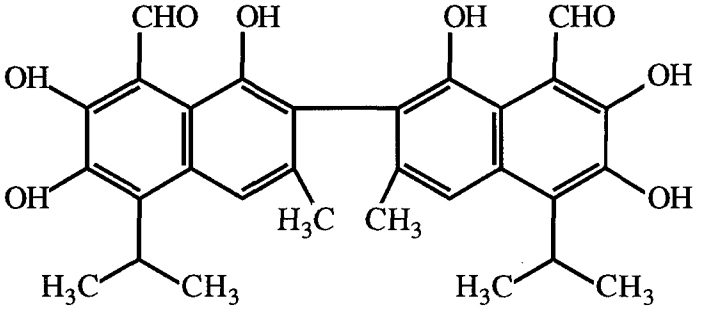


707C91



764C89

567C91: 3-(3,4-dimethoxyphenyl)-2-(3-pyridyl)acrylonitrile
690C88: α-cyano-4-hydroxy-3,5-diisopropylcinnamamide
707C91: 2-cyano-3-(3,4-dihydroxyphenyl)-N-(3-phenylpropyl)acrylamide
764C89: α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide



Gossypol

Gossypol: [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-dicarboxyaldehyde]

Figure 6. Structure of cinnamic acid derivatives and gossypol.

Messiha [1991a,b] has reported that gossypol, a polyphenolic aldehyde, Figure 6, inhibits "hepatic aldehyde dehydrogenase activity" when given to mice. Again, the sensitivity of class 3 aldehyde dehydrogenases to this agent was not tested, thus, we did so.

The ALDH-3 present in human tumor cells/tissues (tALDH-3), e.g., cultured breast adenocarcinoma MCF-7 cells, colon carcinoma C cells, and salivary gland Warthin tumors and mucoepidermoid carcinomas, although otherwise seemingly identical to the ALDH-3 present in human normal tissues/fluids (nALDH-3), e.g., stomach mucosa and saliva, differs from the latter in that it exhibits a much greater ability to catalyze the oxidative detoxification of the oxazaphosphorines [Sladek et al., 1995; Sreerama and Sladek, 1996] and a much greater sensitivity to the inhibitory action of several chlorpropamide analogues [Devaraj et al., 1997; last year's progress report]. Hence, both tALDH-3 and nALDH-3 were included in our investigations.

Human ALDH-1, known to also catalyze the irreversible oxidation (detoxification of aldophosphamide [Dockham et al., 1992], and human ALDH-2 were included in our investigations so that the relative specificity, if any, of the inhibitory effect of these agents towards each of the three classes of aldehyde dehydrogenases could be ascertained.

Generation and purification of human recombinant ALDH-1 (rALDH-1) and ALDH-2 (rALDH-2) was as described in last year's progress report and by Devaraj et al. [1997]. Human normal stomach mucosa ALDH-3 (nALDH-3) and the ALDH-3 (tALDH-3) present in human breast adenocarcinoma MCF-7/0 cells cultured in the presence of 30 μM catechol for 5 days to induce the enzyme (MCF-7/0/CAT cells) were purified as described previously [Sreerama and Sladek, 1993b; Sreerama et al., 1995a].

None of the cinnamic acid derivatives proved to be a very potent inhibitor of the human ALDH-3s, nor were either of these enzymes differentially more sensitive to the inhibitory action of these agents, Table 4.

Gossypol proved to be a very potent inhibitor of the human ALDH-3s, Table 5. Moreover, the ALDH-3s were differentially more sensitive to the inhibitory action of gossypol. The inhibitory action of gossypol was competitive with respect to the cofactor, viz., NAD or NADP, and appeared to be irreversible (data not presented).

Aldehyde dehydrogenases are bifunctional enzymes, i.e., they catalyze both oxidative (oxidation of aldehydes to acids) and hydrolytic (hydrolysis of esters) reactions. Thus, the effect of gossypol on aldehyde dehydrogenase-catalyzed hydrolysis was also determined. In no case was 50% inhibition achieved at the highest concentration, 200 uM, of gossypol tested, Table 5.

Table 4. Inhibition by cinnamic acid derivatives of human aldehyde dehydrogenase-catalyzed oxidation: IC_{50} values[a]

Enzyme	IC_{50}, μM			
	567C91	690C88	707C91	764C89
rALDH-1	88	76	60	> 500
rALDH-2	> 250	147	117	> 500
nALDH-3	154	> 500	> 250	> 500
tALDH-3	181	-	144	-

[a]Enzymes were incubated with vehicle or 2 - 5 different concentrations of the putative inhibitor for 5 min, substrate was added, and initial catalytic rates were quantified as described previously [Dockham et al., 1992; Sreerama and Sladek 1993a]. Substrates and cofactors were acetaldehyde and NAD (4 mM each) for rALDH-1, acetaldehyde (2 mM) and NAD (4 mM) for rALDH-2, and benzaldehyde (4 mM) and NAD (1 mM) for the ALDH-3s. Uninhibited catalytic rates (mean; n = 2) were 0.6, 2.1, 31 and 32 IU/mg protein for rALDH-1, rALDH-2, nALDH-3 and tALDH-3, respectively. IC_{50} values (concentrations of the test compounds required to effect 50% inhibition of catalytic activity) were estimated from plots of % control activity vs inhibitor concentration.

Thus, gossypol exhibited all of the properties that we were looking for, viz., a potent and relatively specific, irreversible inhibitor of the ALDH-3s, and it was, therefore, submitted to further testing (see task # 4).

Also submitted to further testing were two analogues of chlorpropamide, viz., NPI-2 and API-2, Figure 7. The rationale for the design of these and several other analogues of chlorpropamide was given in last year's progress report as were some of our initial findings. Synthesis of these agents was by the laboratory of Dr. H. T. Nagasawa as described previously [Lee et al., 1922a,b]. Exhaustive experimentation during the past year essentially confirmed the initial findings and established that, of the five chlorpropamide analogues investigated, NPI-2 and API-2 showed the most promise as selective *in vivo* inhibitors of ALDH-3, Table 6. Although NPI-2 was only moderately potent with regard to inhibiting tALDH-3 (IC_{50} = 22 μM), tALDH-3 was differentially much more sensitive to inhibition effected by NPI-2 and the inhibition appeared to be irreversible (data not presented). Inhibition of tALDH-3 by API-2 was effected at a very low concentration (IC_{50} = 0.75 μM) but appeared to be easily reversible (data not presented) and was not entirely specific for tALDH-3 since rALDH-2 was approximately ten times more sensitive to this agent. Thus, at first glance, API-2 would not appear to have any future as a clinically useful inhibitor of tALDH-3 since it would inhibit ALDH-2 to an even greater extent. In fact, this is not the case because ALDH-2 is an enzyme that humans can apparently do without since 30-50% of

Orientals lack a functional ALDH-2 and do not suffer any recognized ill-effects as a consequence thereof except for those following the ingestion of alcohol [Goedde and Agarwal, 1990].

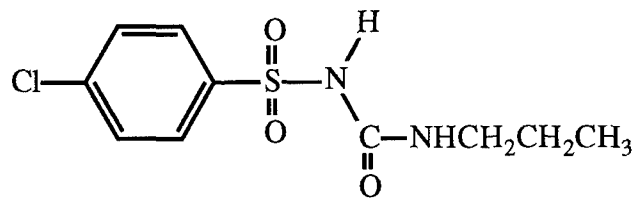
Table 5. Inhibition by gossypol of human aldehyde dehydrogenase-catalyzed oxidation and hydrolysis: IC_{50} values[a]

Enzyme	IC_{50}, μM	
	Oxidation[b]	Hydrolysis[c]
rALDH-1	75	> 200
rALDH-2	45	> 200
nALDH-3	7.5	> 200
tALDH-3	6.6	> 200
GAPDH	70	—
Alkaline phosphatase	—	130

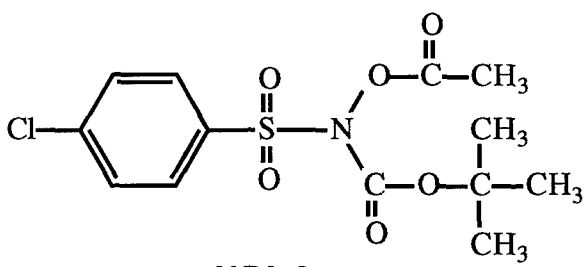
[a]Enzymes were incubated with vehicle or various concentrations of gossypol for 5 min, substrate was added, and initial catalytic rates were quantified as described previously [Rekha and Sladek, 1997a]. A minimum of seven gossypol concentrations, at least two of which effected less than 50% inhibition, and two of which effected greater than 50% inhibition, were used. IC_{50} values (concentrations of the test compounds required to effect 50% inhibition of catalytic activity) were estimated from plots of % control activity vs inhibitor concentration.

[b]Substrate and cofactors were, respectively, acetaldehyde and NAD (4 mM each) for rALDH-1, acetaldehyde (2 mM) and NAD (4 mM) for rALDH-2, benzaldehyde (4 mM) and NAD (1 mM) for the ALDH-3s, and glyceraldehyde 3-phosphate and NAD (1 mM each) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Uninhibited catalytic rates were 0.56, 3.3, 25, 31 and 56 IU/mg protein for rALDH-1, rALDH-2, nALDH-3, tALDH-3 and GAPDH, respectively.

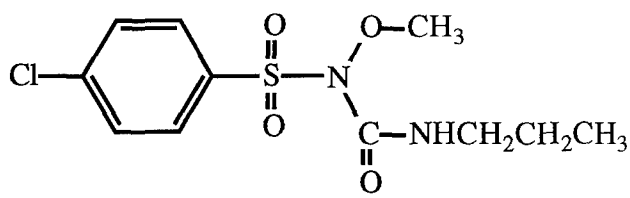
[c]Substrates were *p*-nitrophenyl acetate (500 μM) for the dehydrogenases and *p*-nitrophenyl phosphate (10 mM) for AP. Uninhibited catalytic rates were 126, 476, 11, 9.6 and 16 IU/mg protein for rALDH-1, rALDH-2, nALDH-3, tALDH-3 and alkaline phosphatase, respectively.



Chlorpropamide



NPI-2



API-2

Chlorpropamide: 1-(p-chlorobenzenesulfonyl)-3-*n*-propylurea

NPI-2: (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester

API-2: 4-chloro-N-methoxy-N-[(propylamino)carbonyl]benzenesulfonamide

Figure 7. Structures of chlorpropamide and two analogs thereof.

Table 6. Inhibition by chlorpropamide analogues of human aldehyde dehydrogenases-catalyzed oxidation and hydrolysis: IC_{50} values[a]

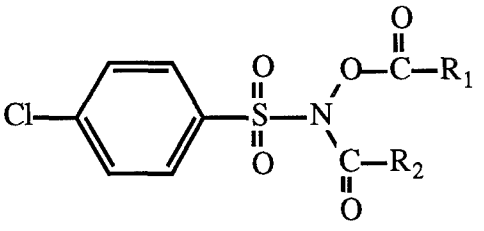
Enzyme	IC_{50}, μM					
	NAD-linked oxidation[b]		NADP-linked oxidation[b]		Hydrolysis[c]	
	NPI-2	API-2	NPI-2	API-2	NPI-2	API-2
rALDH-1	> 400	7.5	—	—	> 200	> 1000
rALDH-2	366	0.08	—	—	> 200	18
nALDH-3	178	5.0	267	0.90	> 200	> 1000
tALDH-3	22	0.75	39	0.62	> 200	> 1000
GAPDH	111	38	—	—	—	—
Alkaline phosphatase	—	—	—	—	> 400	> 1000

[a]Enzymes were incubated with vehicle or 5 - 8 different concentrations of one of the putative inhibitors for 5 min, substrate was added, and initial catalytic rates were quantified as described previously [Devaraj et al., 1997]. IC_{50} values (concentrations of the test compounds required to effect 50% inhibition of catalytic activity) were estimated from plots of % control activity vs inhibitor concentration.

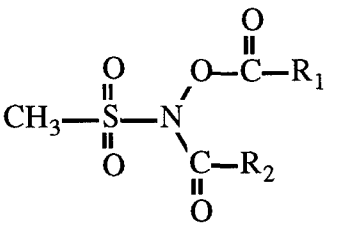
[b]Substrates and cofactors were, acetaldehyde and NAD (4 mM each) for rALDH-1, acetaldehyde (2 mM) and NAD (4 mM) for rALDH-2, benzaldehyde (4 mM) and NAD (1 mM) or NADP (4 mM) for the ALDH-3s, and glyceraldehyde-3-phosphate and NAD (1 mM each) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Uninhibited catalytic rates (mean; n = 2) were 0.59, 2.1, 31, 53, 32, 52 and 50 IU/mg protein for rALDH-1, rALDH-2, NAD-linked nALDH-3, NADP-linked nALDH-3, NAD-linked tALDH-3, NADP-linked tALDH-3 and GAPDH, respectively.

[c]Substrates were *p*-nitrophenyl acetate (500 μM) for the dehydrogenases and *p*-nitrophenyl phosphate (10 mM) for alkaline phosphatase. Uninhibited catalytic rates (mean; n = 2) were 147, 578, 8.7, 9.5 and 15 IU/mg protein for rALDH-1, rALDH-2, nALDH-3, tALDH-3 and alkaline phosphatase, respectively.

In addition to the five chlorpropamide analogues already tested, Dr. Nagasawa's laboratory designed and synthesized two additional series of NPIs (nitroxyl proinhibitors) based on prototype compounds **1** and **2,** Figure 8. Whether any of these agents inhibit any of the aldehyde dehydrogenases remains to be determined.



1



2

1a $R_1 = CH_3$, $R_2 = C_6H_5$
1b $R_1 = R_2 = OCH_2CH_3$

1d $R_1 = R_2 = OC(CH_3)_3$

2a $R_1 = R_2 = OCH_3$
2b $R_1 = R_2 = OCH_2CH_3$
2c $R_1 = R_2 = OCH_2CH_2CH_3$
2d $R_1 = R_2 = OC(CH_3)_3$
2e $R_1 = R_2 = OC_6H_5$

Figure 8. Analogs of chlorpropamide.

Task # 4: Evaluate identified inhibitors of the relevant class 3 aldehyde dehydrogenase activity with respect to their ability to sensitize our oxazaphosphorine-resistant models to the oxazaphosphorines.

The ability of two chlorpropamide analogues, viz., NPI-2 and API-2, and gossypol to negate the influence of relatively high cellular levels of ALDH-3 on the cellular sensitivity of tumor cells (cultured human breast adenocarcinoma MCF-7/0/CAT cells) to oxazaphosphorines was determined for the reasons given under task # 3.

Addition of NPI-2, API-2 or gossypol to the drug-exposure medium prior to exposure to mafosfamide markedly increased the sensitivity of tumor cells that express large amounts of ALDH-3, viz., MCF-7/0/CAT, to the oxazaphosphorine, Figure 9 and Table 7. As expected, identical treatment of tumor cells that express very small amounts of ALDH-3, viz., MCF-7/0, only very minimally increased their sensitivity to mafosfamide. Also as expected because ALDH-3 does not catalyze the detoxification of phosphoramide mustard, the ultimate cytotoxic metabolite of mafosfamide [Sladek, 1994], addition of NPI-2, API-2 or gossypol to the drug-exposure medium prior to exposure to this agent essentially did not increase the sensitivity of MCF-7/0/CAT cells to it, Table 7.

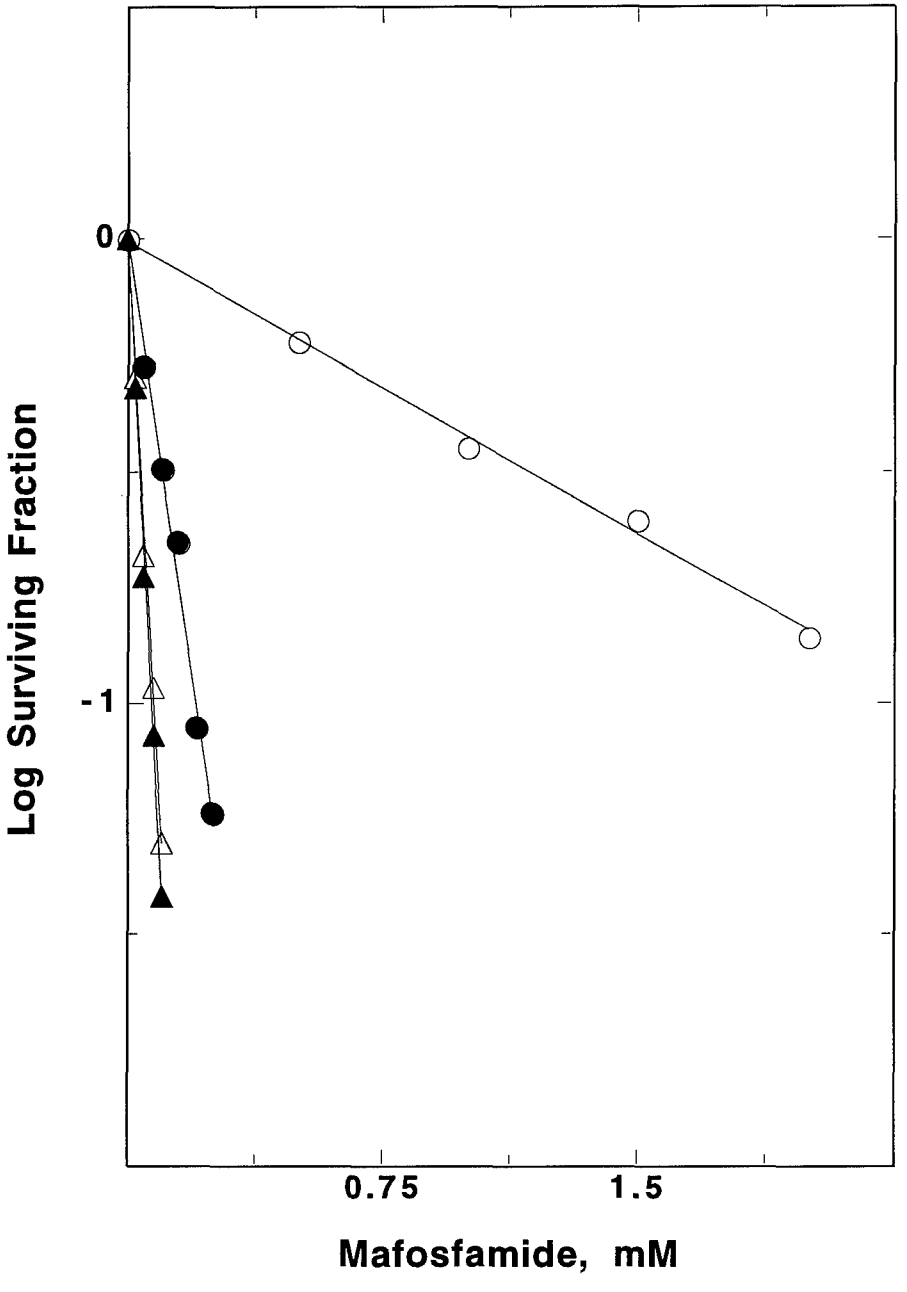


Figure 9. Sensitivities of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide in the presence and absence of gossypol. Exponentially growing MCF-7/0 cells were cultured in the presence of vehicle (△, ▲; MCF-7/0) or 30 μM catechol (○, ●; MCF-7/0/CAT) for 5 days after which time they were each harvested and incubated with vehicle (△, ○) or 75 μM gossypol (▲, ●) for 5 min at 37°C. Mafosfamide was added and incubation was continued for an additional 30 min. The cells were then harvested and grown in drug-free growth medium for 15 days. The colony-forming assay described in Sreerama and Sladek [1993a] was used to determine surviving fractions. Data points are means of triplicate determinations. Cellular levels of aldehyde dehydrogenase activities (cofactor and substrate were 4 mM each of NADP and benzaldehyde, respectively) were 1.8 and 680 mIU/10^7 cells in 105,000 g supernatant fractions obtained from Lubrol-treated whole homogenates of MCF-7/0 and MCF-7/0/CAT cells, respectively.

Table 7. Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide and phosphoramide mustard in the presence and absence of inhibitors of ALDH-3[a]

Cell Line	ALDH-3 (mIU/10^7 cells)	Inhibitor	LC_{90}, μM	
			Mafosfamide	Phosphoramide Mustard
MCF-7/0	2	None	65	800
		NPI-2	60	*b*
		API-2	60	*b*
		Gossypol	65	*b*
MCF-7/0/CAT	665	None	> 2000	1350
		NPI-2	175	1300
		API-2	200	1400
		Gossypol	200	1100

[a]Human breast adenocarcinoma MCF-7/0 cells were cultured in the presence of vehicle (MCF-7/0) or 30 μM catechol (MCF-7/0/CAT) for 5 days. At the end of this time, cells were harvested, washed, and resuspended in drug-exposure medium. The cells (1 x 10^5 cells/ml) were then incubated with NPI-2 (100 μM), API-2 (50 μM), gossypol (75 μM) or vehicle for 5 min at 37°C after which time various concentrations of mafosfamide, phosphoramide mustard or vehicle were added and incubation was continued as before for 30 min at 37°C. The colony-forming assay described previously [Sreerama and Sladek, 1993a] was used to determine surviving fractions. LC_{90} values (concentrations of drug required to effect 90% cell-kills) were obtained from plots of log surviving fractions versus concentrations of drug, Figure 9. Values are means of LC_{90}s obtained in two experiments. Cellular levels of ALDH-3 activity (NADP-linked enzyme-catalyzed oxidation of benzaldehyde; 4 mM each of cofactor and substrate) in 105,000 g supernatant fractions obtained from Lubrol-treated whole homogenates of tumor cells were determined as described previously [Sreerama and Sladek, 1993a].

[b]Not determined.

The findings reported herein establish the therapeutic potential of combining NPI-2, API-2 or gossypol with an oxazaphosphorine in the treatment of certain cancers. Uncertain is whether these agents will inhibit tALDH-3 *in vivo* at doses that do not cause untoward effects since this possibility remains essentially untested. Experiments with a limited number of animals showed that NPI-2, 1 mmol/kg, ip, did not inhibit aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde in rats, as judged by its failure to cause elevated plasma levels of acetaldehyde in animals given ethanol [Lee et al., 1992b]. The aldehyde dehydrogenases that are thought to catalyze the bulk of acetaldehyde oxidation *in vivo,* viz., class 2, and to a lesser extent class 1, aldehyde dehydrogenases, are not very sensitive to the inhibitory action of NPI-2,

Table 6. In contrast, the class 3 aldehyde dehydrogenases, especially tALDH-3, are, Table 6. Thus, the possibility that tolerated doses of NPI-2 will inhibit tALDH-3 *in vivo* remains viable. API-2, 1 mmol/kg, ip, on the other hand, markedly inhibited aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde in rats, as judged by the markedly elevated plasma levels of acetaldehyde that were observed when the animals were treated with this agent prior to being given ethanol [Lee et al., 1992a].

Gossypol has been shown to effect male contraception and *in vitro* tumor cell-kill [Tuszynski and Cossu, 1984; Joseph et al., 1986; Band et al., 1989 Wu, 1989; Wu et al., 1989; Benz et al., 1990; Ford et al., 1991; Hu et al., 1993; Coyle et al., 1994; Gilbert et al., 1995] More importantly, it was demonstrated in several animal models that tumor cell-kill could be achieved *in vivo* with doses of gossypol that were not injurious to the host animal [Tso, 1984; Wu et al., 1989; Chang et al., 1993; Naik et al., 1995]. These observations prompted phase 1, and, subsequently, phase 2, clinical trials of gossypol for the treatment of certain cancers, viz., metastatic carcinomas of the ovary [Wu, 1989], various advanced cancers [Stein et al., 1992], metastatic adrenocortical carcinomas [Flack et al., 1993] and metastatic breast cancers [Seidman, 1996]. Thus, much is known about the pharmacokinetic behavior and toxicity of gossypol in humans [Wu, 1989]. Whether doses of gossypol sufficient to inhibit tALDH-3 *in vivo* can be given to humans safely remains to be determined but seems probable given the foregoing and that the concentration of gossypol needed to inhibit tALDH-3 *in vitro* (IC_{50} = about 7 μM; Table 5) is much less than that needed to inhibit tumor cell proliferation in our *in vitro* model, viz., cultured human breast adenosarcoma MCF-7 cells (LC_{90} = about 200 μM; data not presented). Indeed, in this model, complete negation of tALDH-3-mediated oxazaphosphorine resistance is effected by a concentration of gossypol, viz., 75 μM or less, that is not toxic to MCF-7 cells, Figure 9 and Table 7. In any event, our findings establish the therapeutic potential of combining gossypol with an oxazaphosphorine in the treatment of certain cancers. Moreover, given the antitumor activity that gossypol itself exhibits, it can be envisasged that in the case of some of these cancers, gossypol could be of dual therapeutic value when combined with an oxazaphosphorine in the therapeutic protocol.

Task # 5: Identify the molecular basis for the apparent overexpression of class 3 aldehyde dehydrogenases in our model systems.

We have yet to address this task.

Task # 6: Ascertain the ability of Ah receptor ligands to induce class 3 aldehyde dehydrogenase overexpression and oxazaphosphorine-specific acquired resistance in estrogen receptor-positive and -negative breast cancer cell lines that lack and express Ah receptors.

We previously noted [Sreerama and Sladek, 1994; Sladek et al., 1995] that PAHs induced ALDH-3 and oxazaphosphorine-specific resistance in breast cancer cells that were, reportedly, estrogen receptor-

positive, e.g., MCF-7/0, T-47D and ZR-75-1 (all, reportedly, Ah receptor-positive), but not in those that were, reportedly, estrogen receptor-negative, e.g., MDA-MB-231 (reportedly, Ah receptor-positive) and SK-BR-3 (Ah receptor status unknown) [Engel and Young, 1978; Vickers et al., 1989; Safe et al., 1991; Taylor-Papadimitriou et al., 1993]. In contrast, phenolic antioxidants induced ALDH-3 and oxazaphosphorine-specific resistance in both estrogen receptor-positive and estrogen receptor-negative cells [Sladek et al., 1995; Sreerama et al., 1995a and unpublished observations].

Updating the above, we have now confirmed that the MCF-7/0, T-47D and ZR-75-1 cell lines that we carry are indeed estrogen and Ah receptor-positive, that the MDA-MB-231 cell line that we carry is indeed estrogen receptor-negative and Ah receptor-positive, and that the SK-BR-3 cell line that we carry is indeed estrogen receptor-negative, Table 8. Additionally, we have established that the MCF-7/OAP cell line that we carry is estrogen and Ah receptor-positive, that the HST-578-T cell line that we now also carry is estrogen receptor-negative and Ah receptor-positive, and that the MDA-MB-435 cell line that we now also carry is estrogen receptor-negative. Finally, we have shown that PAHs, e.g., 3-methylcholanthrene, as well as phenolic antioxidants, e.g., catechol, induce ALDH-3 in MCF-7/OAP cells. Thus far, then, our findings are consistent with our original hypothesis, viz., Ah receptor ligands, e.g., 3-methylcholanthrene, will, via xenobiotic responsive elements (XRE) present in the 5'-upstream region of the ALDH-3 gene, induce ALDH-3 overexpression in estrogen receptor-positive breast cancer cells but not in estrogen receptor-negative breast cancer cells, whereas, induction of ALDH-3 effected by agents that cause the activation of the so-called antioxidant responsive element (ARE) present in the 5'-upstream region of the ALDH-3 gene is estrogen receptor-independent.

Table 8. Receptor status and the ability of methylcholanthrene and catechol to induce cytosolic class 3 aldehyde dehydrogenase activity in human breast (adeno)carcinoma cell lines[a]

Cell Line	Constitutive ALDH-3	Receptor Status[b]		ALDH-3 & OSAR[c] Induced By	
		Ah	Estrogen	MC	CAT
MCF-7/0	+	+	+	Yes	Yes
MCF-7/OAP	+++	+	+	Yes	Yes
T-47D	+	+	+	Yes	Yes
ZR-75-1	+	+	+	Yes	Yes
MDA-MB-231	+	+	-	No	Yes
SK-BR-3	+	?	-	No	Yes
HS-578-T	?	+	-	?	?
MDA-MB-435	?	?	-	?	?

[a]Composite of information heretofore unpublished and data published in Sreerama and Sladek [1993a,b, 1994], Rekha et al. [1994] and Sladek et al. [1995]. Cells were grown (monolayer) in the presence of 3 μM methylcholanthrene (MC) or 30 μM catechol (CAT) for 5 days and harvested. Cytosolic class 3 aldehyde dehydrogenase (ALDH-3) activity (NADP-linked enzyme-catalyzed oxidation of benzaldehyde by Lubrol-treated whole homogenates) was quantified as previously described [Sreerama and Sladek, 1993a]. Semiquantification of estrogen receptor levels was by an enzyme-linked immunosorbent assay (ELISA) as described by Hornbeck et al. [1991]. Semiquantification of Ah receptor levels was esentially as described by Harris et al. [1989] except that ^{3}H-labeled benzpyrene, insted of ^{3}H-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, was used as the receptor ligand.

[b]As per our findings in the cells we grow and in agreement with literature reports [Engel and Young, 1978; Harris et al., 1989; Vickers et al., 1989; Safe et al., 1991; Taylor-Papadimitriou et al., 1993].

[c]Oxazaphosphorine-specific acquired resistance.

Task # 7: Ascertain the ability of ligands for ARE to induce ALDH-3 activity and oxazaphosphorine-specific acquired resistance in our model system.

This task, as originally formulated, was completed in year-1 of the grant period. Results of that investigation were summarized in last year's report. Briefly, phenolic antioxidants, already known to induce glutathione S-transferase and/or DT-diaphorase activities in various rodent and human organ/tissue/cells via AREs present in the 5'-upstream regions of these enzymes [reviewed in Talalay et al., 1987; Belinsky and Jaiswal, 1993], were found to rapidly, coordinately and reversibly induce ALDH-3, glutathione S-transferase, DT-diaphorase and UDP-glucuronosyl transferase in cultured breast and other tumor cells, the consequence of which was that such cells became less sensitive to certain anticancer drugs (multidrug resistance) and more sensitive to others (collateral sensitivity). These findings should be viewed as greatly expanding the number of recognized dietary, environmental and pharmaceutical agents that can potentially influence the sensitivity of breast and other tumor cells to cyclophosphamide, other

oxazaphosphorines and still other antitumor agents since, in addition to phenolic antioxidants which themselves are abundantly present in a number of dietary materials, a number of other dietary, etc., agents are thought to effect enzyme induction via the signaling mechanism used by the phenolic antioxidants.

These and other findings in our laboratory serve as the bases for a second-generation investigation that we expect to pursue, at least in a preliminary sense. The hypothesis, rationale therefor, and significance thereof are as follows:

Given that 1) cellular sensitivity to anticancer agents, as well as to potential carcinogens, is known to be highly variable, 2) molecular determinants of cellular sensitivity to certain anticancer agents and certain potential carcinogens include enzymes, e.g., ALDH-3, several glutathione S-transferases and DT-diaphorase, that catalyze their biotransformation to more active, or less active, metabolites [reviewed in Bock et al., 1990; Riley and Workman, 1992; Workman, 1994; Hayes and Pulford, 1995; Rekha and Sladek, 1997b; Sladek et al., 1995], 3) ALDH-3, glutathione S-transferases and DT-diaphorase are transiently induced by agents widely present in the diet or elsewhere in the environment [Talalay et al., 1987; Belinsky and Jaiswal, 1993; Sreerama and Sladek, 1994; Sladek et al., 1995; Sreerama et al., 1995a], 4) each of these enzymes is present in the saliva [Sreerama et al., 1995b], 5) salivary levels of these enzymes are elevated following the ingestion of broccoli or other dietary materials, e.g., coffee, known to contain agents that induce these enzymes [Sreerama et al., 1995b], and 6) cellular levels of these enzymes vary widely in normal and malignant breast tissues (see task # 1), **it follows that** salivary levels of ALDH-3, the glutathione S-transferases and DT-diaphorase may reflect normal and tumor tissue levels of these enzymes and that, **therefore**, salivary levels of ALDH-3, the glutathione S-transferases and/or DT-diaphorase would be prognostic indicators of tumor cell sensitivity to certain anticancer agents and of the chemopreventive potential of certain agents/diets. Attractive is the non-invasiveness of sample collection.

The essence of the preliminary investigation will be to quantify ALDH-3, glutathione S-transferase and DT-diaphorase levels in a limited number of presurgery saliva samples and in subsequently surgically removed normal and malignant breast tissues and then to analyze the resultant data with regard to any correlations between salivary enzyme levels and the corresponding breast tissue enzyme levels.

CONCLUSIONS

ALDH-3 and ALDH-1 catalyze the detoxification of oxazaphosphorines. In each case, detoxification is effected when the enzyme catalyzes the oxidation of aldophosphamide to carboxyphosphamide. Elevated levels of either of these enzymes in cultured tumor cell models account for the decrease in cellular sensitivity to the oxazaphosphorines exhibited by such models. The broad range of ALDH-3 and ALDH-1 levels found in surgically removed human breast tumor samples indicates that variable levels of these enzymes accounts, at least in some cases, for the variable response of breast tumors to cyclophosphamide and other oxazaphosphorines in the clinic.

A broad range of glutathione S-transferase and DT-diaphorase, as well as of ALDH-3 and ALDH-1, levels was found in human breast tumor tissue samples. Glutathione S-transferases catalyze the detoxification of a number of chemotherapeutic agents, e.g., melphalan and chlorambucil. DT-diaphorase catalyzes the activation of at least two chemotherapeutic agents, viz., mitomycin C and EO9. Thus, the broad range of glutathione S-transferase and DT-diaphorase levels found in surgically removed human breast tumor samples indicates that variable levels of these enzymes accounts, at least in some cases, for the variable response of breast tumors to these agents in the clinic.

As judged by *in vitro* experiments, at least three agents, viz., gossypol and two chlorpropamide derivatives, are of potential value *in vivo* with regard to relatively selectively inhibiting ALDH-3-catalyzed oxidative reactions, thereby sensitizing tumor cells, otherwise insensitive to cyclophosphamide and other oxazaphosphorines because they express large amounts of ALDH-3, to these agents. Moreover, human tumor cell ALDH-3 was found to be more sensitive to each of the chlorpropamide analogues than was human normal cell ALDH-3. Thus, it may be possible to develop a clinically useful selective inhibitor of tumor cell ALDH-3 thereby allowing the selective sensitization of tumor cells expressing large amounts of ALDH-3 to cyclophosphamide and other oxazaphosphorines.

Abundantly present dietary/environmental constituents coordinately induce ALDH-3, several glutathione S-transferases, DT-diaphorase, UDP-glucuronosyl transferase and other enzymes in our cultured human breast cancer models. As stated above, these enzymes catalyze the biotransformation (detoxification in most cases; toxification in some) of cancer chemotherapeutic agents. Induction is reversible. Supporting the notion of coordinated induction by dietary and other agents of ALDH-3, glutathione S-transferase and DT-diaphorase *in vivo*, e.g., in human breast tumor tissue, was the fact that, in some cases, the levels of all three were elevated in the repository samples that we examined. Thus, attention to the composition of the diet may be prudent, and even used advantageously, when using relevant cancer chemotherapeutic agents.

As judged by the information that we have generated thus far, agents that induce ALDH-3, several glutathione S-transferases, DT-diaphorase, UDP-glucuronosyl transferase and other enzymes via Ah

receptors and XREs present in the 5'-upstream regions of these enzymes e.g., polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, can only do so in estrogen-receptor positive cells, i.e., they cannot induce them in estrogen receptor-negative cells, whereas agents that induce ALDH-3, several glutathione S-transferases, DT-diaphorase, UDP-glucuronosyl transferase and other enzymes via AREs present in the 5'-upstream regions of these enzymes, e.g., antioxidants such as catechol, can do so in both estrogen receptor-positive and -negative cells.

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*Included in the appendix (reprints enclosed).

APPENDIX
(Reprints Enclosed)

Devaraj, V. R., Sreerama, L., Lee, M. J. C., Nagasawa, H. T. and Sladek, N. E. Yeast aldehyde dehydrogenase sensitivity to inhibition by chlorpropamide analogues as an indicator of human aldehyde dehydrogenase sensitivity to these agents. *In*: Weiner, H., Lindahl, R., Crabb, D.W. and Flynn, T.G. (eds.), *Enzymology and Molecular Biology of Carbonyl Metabolism - 6*, New York: Plenum Press, 1997, In press.

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YEAST ALDEHYDE DEHYDROGENASE SENSITIVITY TO INHIBITION BY CHLORPROPAMIDE ANALOGUES AS AN INDICATOR OF HUMAN ALDEHYDE DEHYDROGENASE SENSITIVITY TO THESE AGENTS

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INTRODUCTION

Aldehyde dehydrogenase (E.C. 1.2.1.3) is a polymorphic enzyme that is relatively substrate-nonspecific. Several isoenzymes are found in human tissues. Based on primary structure or subcellular distribution and kinetic, physical and immunochemical properties, they have been placed into one of three classes, viz., class 1, e.g., ALDH-1, class 2, e.g., ALDH-2 and class 3, e.g., ALDH-3 (Anonymous, 1989; Lindahl and Hempel, 1990; Goedde and Agarwal, 1990; Lindahl, 1992). These enzymes catalyze the biotransformation (bioactivation and/or bioinactivation) of a broad spectrum of endogenous (biogenic) and exogenous (xenobiotic) aldehydes that are physiologically and/or pharmacologically important (Sladek et al., 1989, 1995; Lindahl, 1992; Sladek, 1993, 1994). For example, ALDH-1 catalyzes the oxidation of retinaldehyde to retinoic acid, the latter being a potent modulator of cell growth and differentiation; ALDH-1 and, especially, ALDH-2 catalyze the oxidation of ethanol-derived acetaldehyde to acetate, a detoxifying reaction; ALDH-3 catalyzes the oxidation of 4-hydroxynonenal and other aldehydic products of lipid peroxidation to their corresponding acids, also a detoxifying reaction; and ALDH-1 and ALDH-3 catalyze the oxidation of aldophosphamide to carboxyphosphamide, yet another detoxifying reaction, since, alternatively, aldophosphamide, a metabolite of anticancer prodrugs collectively known as oxazaphosphorines, e.g., cyclophosphamide, gives rise to phosphoramide mustard, the metabolite that effects the cytotoxic action of these prodrugs.

It follows that inhibitors of these enzymes would be of experimental, and, in some cases, even of clinical, value. Inhibitors of ALDH-1 and ALDH-2, e.g., chloral hydrate, disulfiram and cyanamide, have been identified, and, in the case of disulfiram and cyanamide, their clinical utility as alcohol deterrents has been established (Peachey, 1981; Sellers et al., 1981; Brien and Loomis, 1985; Petersen, 1992). However, these agents do not inhibit ALDH-3 *in vitro* or *ex vivo* at pharmacologically relevant concentrations (Sreerama and Sladek, 1993, 1994; Sladek et al., 1995) and, to date, there are, with the exception of gossypol (Rekha and Sladek, 1997), no known inhibitors of ALDH-3, although alternative substrates, e.g., benzaldehyde and diethylaminobenzaldehyde, can be used to inhibit ALDH-3-catalyzed oxidation of other aldehydes, e.g., aldophosphamide (Sreerama and Sladek, 1993, 1994, 1995; Rekha et al., 1994; Sladek et al., 1995; Bunting and Townsend, 1996).

Like disulfiram and cyanamide, the oral hypoglycemic agent chlorpropamide is thought to be a pro-inhibitor of the aldehyde dehydrogenases, most notably ALDH-2, that catalyze the oxidation of ethanol-derived acetaldehyde (Öhlin et al., 1982; Little and Petersen, 1985). Consistent with this notion, about 23% of patients who receive this agent experience, on ingesting alcohol, an adverse reaction characterized by facial flushing and general malaise. These reactions are identical to those experienced by persons who consume alcohol following administration of disulfiram as well as by functional ALDH-2-deficient individuals who consume alcohol (Logie et al., 1976; Öhlin et al., 1982; Goedde and

Agarwal, 1990; Petersen, 1992). Nitroxyl (HNO) and *n*-propylisocyanate have been postulated to be the chlorpropamide metabolites that inhibit hepatic aldehyde dehydrogenase-catalyzed reactions (Nagasawa et al., 1988, 1989; Lee et al., 1992a,b).

Based on this premise, a number of N^1-substituted chlorpropamide analogues, viz., ester and alkyl derivatives, have been designed and synthesized as potential alcohol deterrents (Nagasawa et al., 1988, 1989; Lee et al., 1992a,b).

Aldehyde dehydrogenases are bifunctional enzymes in that they catalyze the hydrolysis of esters in addition to catalyzing the oxidation of aldehydes (Feldman and Weiner, 1972; Sidhu and Blair, 1975; Blatter et al., 1992; Sladek et al., 1995). Whether catalysis of hydrolytic reactions by these enzymes is of physiological or pharmacological consequence is not known.

The ester analogues were designed with the intent of exploiting the dual catalytic activities exhibited by the aldehyde dehydrogenases, viz., to release HNO, a potent inhibitor of aldehyde dehydrogenase-catalyzed oxidations, upon ester hydrolysis catalyzed by these enzymes (Lee et al., 1992b). Indeed, some of the compounds that were synthesized have been shown to undergo hydrolytic cleavage catalyzed by yeast aldehyde dehydrogenase (yALDH) and to inhibit yALDH-catalyzed acetaldehyde oxidation.

The alkyl analogues, on the other hand, were designed to release *n*-propylisocyanate, a potent inhibitor of yeast and rodent hepatic aldehyde dehydrogenases, without the necessity of any enzyme participation (Nagasawa et al., 1988, 1989; Lee et al., 1992a). Some of the compounds that were synthesized have been shown to inhibit yeast and rodent hepatic mitochondrial aldehyde dehydrogenase-catalyzed oxidation, decrease acetaldehyde clearance in rodents given ethanol, and to be devoid of a hypoglycemic effect.

Historically, we and others have used the ability of these and other compounds to inhibit yALDH-catalyzed oxidation of acetaldehyde as the initial indicator of potentially useful alcohol-deterrent activity (Watanabe et al., 1986; Nagasawa et al., 1988, 1989; Lee et al., 1992a,b). Although the catalytic site amino acids are conserved (Saigal et al., 1991), yeast and human aldehyde dehydrogenases differ substantially with regard to their primary structure and catalytic properties (Bostian and Betts, 1978; Lindahl and Hempel, 1990). Thus, the sensitivity of yALDH to a candidate inhibitor may not be paralleled by the sensitivity of human aldehyde dehydrogenases to it, and the use of yALDH to screen for potential alcohol deterrents may not be predictive. Alternatives to the use of yALDH in the initial screen, albeit not commercially available and costlier to generate, are recombinant human ALDH-1 and ALDH-2 (rALDH-1 and rALDH-2, respectively).

The present investigation sought to ascertain whether sensitivity to a candidate inhibitor on the part of ALDH-1 and/or ALDH-2, and, for completeness, ALDH-3, was in fact, predicted by the sensitivity of yALDH to inhibition by that agent. Further, since the ALDH-3 present in human normal tissues/fluids (nALDH-3) is somewhat different from the ALDH-3 present in human tumor cells/tissues (tALDH-3), viz., the latter (putatively, tumor-specific) is better able to catalyze the detoxification of aldophosphamide (Sladek et al., 1995; Sreerama and Sladek, 1995), both nALDH-3 and tALDH-3 were included in our investigations. Chlorpropamide analogues of the types described above (Fig. 1) were used as test inhibitors.

"Figure" 1. Structures of chlorpropamide, three analogues thereof, and Piloty's acid.

MATERIALS AND METHODS

E. coli [BL21(DE3)pLysS] transfected with pET-19b vector to which human ALDH-1 cDNA [cloned from human hepatoma Hep G2 cells; cDNA sequence was identical to that of human liver ALDH-1 cDNA (Moreb et al., 1996)] was ligated, was provided by Dr. Jan Moreb, University of Florida, Gainsville, FL. A vector, viz., pT7-7, to which human ALDH-2 cDNA [cloned from human liver (Zheng et al., 1993)] was ligated, was provided by Dr. Henry Weiner, Purdue University, Lafayette, IN. Transfection of human ALDH-2 cDNA ligated to the pT7-7 vector into *E. coli* [BL21(DE3)pLysS] was by Drs. P. A. Dockham and L. Sreerama of our laboratory as described by Sambrook et al. (1989). (Benzoyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester (NPI-1), N-acetyl-N-(acetyloxy)-4-chlorobenzenesulfonamide (NPI-3) and 4-chloro-N-ethyl-N-[(propylamino)carbonyl]benzenesulfonamide (API-1) were synthesized as described previously (Nagasawa et al., 1989; Lee et al., 1992b). Chromatographically purified yeast aldehyde dehydrogenase, human erythrocyte glyceraldehyde-3-phosphate dehydrogenase, human placental alkaline phosphatase type XXIV, chlorpropamide, *p*-nitrophenyl phosphate, phenylmethylsulfonylfluoride and Triton X-100 (*t*-octylphenyloxypolyethyloxyethanol) were purchased from Sigma Chemical Co., St. Louis, MO. Benzenesulfohydroxamic acid (Piloty's acid) was purchased from Aldrich Chemical Co., Milwaukee, WI. Ampicillin, chloramphenicol and isopropylthio-β-D-galactoside were purchased from USB Corp., Cleveland, OH. Centriprep-30 concentrators were purchased from Amicon Inc., Beverly, MA. Ni^{2+}-chelated Sepharose CL 6B (His-Bind resin) was purchased from Novagen Inc., Madison, WI. Luria-Bertani medium (powder) was purchased from Bio-101 Inc., Vista, CA. All other chemicals and reagents were purchased from the sources listed in previous publications (Dockham et al., 1992; Sreerama and Sladek, 1993).

E. coli transfected with ALDH-1 cDNA ligated to the pET-19b vector, or with ALDH-2 cDNA ligated to the pT7-7 vector, were cultured overnight (14-16 h) at 37°C in growth medium, viz., Luria-Bertani broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with ampicillin (50 and 100 mg/L, respectively, in the cases of pT7-7 and pET-19b transfected *E. coli*) and chloramphenicol (34 mg/L), subjected to vigorous shaking (220 rpm) effected by an environmental orbital-shaker (Model 4628, Lab-Line Instruments Inc., Melrose Park, IL) at 37°C. Media absorbance at 600 nm after the overnight culture was usually ~1. The overnight cultures were diluted (1:1) with growth medium, isopropylthio-β–D–galactoside (238 and 95 mg/L, respectively, in the cases of pET-19b and pT7-7 transfected *E. coli*) was added, and the resultant suspensions were cultured for an additional 4 to 5 h at 37°C to induce the expression of rALDH-1 and rALDH-2. *E.coli* were then harvested by centrifugation at 5,000 g and 4°C for 5 min. Bacterial pellets thus obtained were washed (centrifugation) once with 0.9% NaCl after which they were resuspended in a volume of lysis buffer (50 mM Tris-HCl, pH 8.0, supplemented with 2 mM EDTA, 1% Triton X-100 and 10 μM phenylmethylsulfonylfluoride) that was one tenth of the original culture volume and then lysed in an ice-bath by submitting them to sonication (Artek Dismembrator Model 300; setting of 30) for a total of 4 min (divided into 8 bursts interspersed with 1 min rest intervals). The resultant lysates were centrifuged at 5,000 g and 4°C for 10 min and aldehyde dehydrogenase activity was quantified in the supernatant fractions thus obtained. Ion exchange and affinity column chromatography was used as described below to purify the rALDH-1 and rALDH-2 present in these supernatant fractions to apparent homogeneity.

All column chromatographic procedures were performed at 4-6°C. All buffers were degassed prior to use. The protein content of the preparations loaded onto the

chromatographic columns never exceeded 15 mg/ml and typically was much less. Concentration of eluates was with the aid of an Amicon Diaflo concentrator fitted with a YM-30 membrane and pressurized with nitrogen (Dockham et al., 1992; Sreerama and Sladek, 1993) except when the sample volumes were ~10 ml or smaller, in which cases, Amicon Centriprep-30 centrifugation concentrators were used. Acetaldehyde (4 and 2 mM for rALDH-1 and rALDH-2, respectively) and NAD (4 mM) were used as substrate and cofactor, respectively, to monitor rALDH-1 or rALDH-2 activity in column eluates. Protein elution from the columns was monitored at 280 nm with the aid of an ISCO UA-5 absorbance monitor. Otherwise, the method of Bradford (1976) was used to quantify protein concentrations. The Bio-Rad protein assay reagent was used for this purpose; bovine serum albumin was used as the standard.

PD-10 (Sephadex G-25) columns were used as described previously (Dockham et al., 1992; Sreerama and Sladek, 1993) to first transfer the rALDH-1 present in *E. coli* lysate supernatant fractions into a 20 mM imidazole buffer solution, pH 6.8, supplemented with 1 mM each of EDTA and dithiothreitol, and the rALDH-2 present in *E. coli* lysate supernatant fractions into a 25 mM 2-(N-morpholino)ethane sulfonic acid buffer solution, pH 6.0, supplemented with 1 mM each of EDTA and dithiothreitol. DEAE-Sephacel anion exchange chromatography (Dockham et al., 1992) followed by Ni^{2+}-chelated Sepharose CL 6B affinity chromatography (manufacturers [Novagen Inc., Madison WI] protocol) was then used to obtain apparently pure rALDH-1. CM-Sepharose CL 6B cation exchange chromatography followed by DEAE-Sephacel anion exchange chromatography and, subsequently, 5'-AMP-Sepharose CL 6B affinity chromatography (Dockham et al., 1992) was then used to obtain apparently pure rALDH-2. Overall recoveries of rALDH-1 and rALDH-2 were 50 to 60% and 25 to 30%, respectively.

pET-19b is designed to add 21 amino acids (amino acids 2 to 11 being histidines) to either the N- or C-terminal end of the protein coded for by the cDNA ligated to it, thereby enabling purification by Ni^{2+}-chelated Sepharose CL 6B affinity chromatography. Thus, rALDH-1, generated and purified as described above, is actually a fusion protein made up of a 21 amino acid peptide attached to the N-terminal end of ALDH-1. Specific activities (acetaldehyde and NAD, 4 mM each, were the substrate and cofactor, respectively) of several batches of purified rALDH-1 ranged from 600 to 780 mIU/mg protein as compared to a specific activity of 2850 mIU/mg protein obtained for the native enzyme (Sreerama and Sladek, 1997). The subunit molecular mass of rALDH-1 was determined to be 53.7 kDa (data not shown) as compared to a molecular mass of 52 kDa for the native enzyme (Sreerama and Sladek, unpublished observation). The Km value of 429 ± 61 μM (mean ± SE; Table 3) defining rALDH-1 catalyzed oxidation of acetaldehyde at 37°C was essentially identical to that, 483 μM, defining native enzyme-catalyzed oxidation of acetaldehyde under identical conditions (Dockham et al., 1992). Specific activities (acetaldehyde, 2 mM, and NAD, 4 mM, were the substrate and cofactor, respectively) of several batches of purified rALDH-2 ranged from 1700 to 2400 mIU/mg protein. The subunit molecular mass was 52 kDa (data not shown). These values are identical to those obtained for the native enzyme (Dockham et al., 1992).

Human normal stomach mucosa ALDH-3 (nALDH-3) and the ALDH-3 (tALDH-3) present in human breast adenocarcinoma MCF-7/0 cells cultured in the presence of 30 μM catechol for 5 days to induce the enzyme (MCF-7/0/CAT cells) were purified as described previously (Sreerama and Sladek, 1993; Sreerama et al., 1995). Specific activities (benzaldehyde, 4 mM, and NAD, 1 mM, were the substrate and cofactor, respectively) of nALDH-3 and tALDH-3 were 31,000 and 34,000 mIU/mg protein, respectively. The subunit molecular mass of each was 54.5 kDa (data not shown). These values are identical

to those previously reported for these enzymes (Sreerama and Sladek, 1993; Sreerama et al., 1995).

Denaturing polyacrylamide gel electrophoresis (10% polyacrylamide gels containing 1% sodium dodecyl sulfate) was used as described previously (Sreerama and Sladek, 1993) to ascertain the purity of each enzyme preparation.

NAD-linked oxidation of acetaldehyde catalyzed by rALDH-1, rALDH-2 and yALDH at 37°C and pH 8.1, NAD-linked oxidation of benzaldehyde catalyzed by nALDH-3 and tALDH-3 at 37°C and pH 8.1, and hydrolysis of *p*-nitrophenyl acetate catalyzed by each of these enzymes at 25°C and pH 7.5, were quantified spectrophotometrically as described previously (Dockham et al., 1992; Sreerama and Sladek, 1993). NAD-linked oxidation of glyceraldehyde-3-phosphate catalyzed by glyceraldehyde-3-phosphate dehydrogenase at 37°C and pH 7.6, and hydrolysis of *p*-nitrophenyl phosphate catalyzed by alkaline phosphatase at 25°C and pH 9.8, were quantified as described by Lambeir et al. (1991) and Chueh et al. (1981), respectively. Preincubation of the putative inhibitor or vehicle together with the complete reaction mixture except for substrate was for 5 min except in the case of Piloty's acid where it was for 10 min. Preincubation temperatures and pHs were the same as incubation temperatures and pHs. All reactions were started by the addition of substrate. Stock solutions of chlorpropamide and its analogues were prepared in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the reaction mixture never exceeded 5%; this concentration of dimethyl sulfoxide did not inhibit any of the enzyme-catalyzed reactions under investigation. Stock solutions of Piloty's acid were prepared in dimethylformamide. Aliquots thereof of the desired size were placed into reaction tubes, dimethylformamide was evaporated with the aid of a stream of nitrogen, and preincubation was initiated by adding the complete reaction mixture minus the substrate.

Computer-assisted unweighted nonlinear regression analysis effected by the STATView statistical program (Brain Power Inc., Calabas, CA) was used to generate the curves that best-fit plots of enzyme activities (% of control) as a function of inhibitor concentrations (a minimum of five, at least two of which effected less than 50% inhibition and two of which effected greater than 50% inhibition) and, subsequently, to estimate the concentration of inhibitor that effected a 50% decrease in catalytic activity (IC_{50}). Double-reciprocal (Lineweaver-Burk) plots of initial catalytic rates as a function of substrate concentrations (at least three and usually five) were used to estimate the Km and Vmax values. Except in the case of rALDH-2, Ki values were determined by plotting the slopes of the lines generated by double-reciprocal (Lineweaver-Burk) plots as a function of inhibitor concentrations. In the case of rALDH-2, Ki values were determined by plotting the reciprocals of initial catalytic rates as a function of inhibitor concentrations (Dixon plots) because Km values were relatively small and, thus, Ki values were difficult to ascertain accurately from Lineweaver-Burk plots. In the case of double-reciprocal (Lineweaver-Burk) plots, computer-assisted Wilkinson weighted linear regression analysis (Wilkinson, 1961) effected by the MacWilkins program (Microsoft, Bellevue, WA) was used to generate the best-fit lines. Computer-assisted unweighted linear regression analysis effected by the STATView statistical, or Kaleida Graph (Synergy Software, Inc., Reading, PA), programs was used to generate best-fit lines for all other straight-line functions.

RESULTS AND DISCUSSION

In agreement with the reports of others (Öhlin et al., 1982; Little and Petersen, 1985), and the suggestion that metabolic activation is required to effect aldehyde dehydrogenase inhibitory activity (Nagasawa et al., 1985, 1989), chlorpropamide did not inhibit any of the enzymes tested (Table 1).

"Table 1". Inhibition by chlorpropamide analogues and Piloty's acid of yeast and human aldehyde dehydrogenase-catalyzed oxidation: IC_{50} values.

Each of the three chlorpropamide analogues tested, viz., NPI-1, NPI-3 and API-1 (Fig. 1), inhibited enzyme-catalyzed oxidation of aldehydes to acids by each of the substrate-nonspecific aldehyde dehydrogenases tested (Fig. 2 and Table 1). Differential potency on the part of the chlorpropamide analogues was observed. In general, the rank order was NPI-1 > API-1 > NPI-3. Differential sensitivity to these inhibitors on the part of the aldehyde dehydrogenases was also observed. Thus, as judged by IC_{50} values, oxidative catalysis by yALDH was quite sensitive to inhibition by all three analogues whereas that by human aldehyde dehydrogenases often was not. In particular, none of the human aldehyde dehydrogenases were very sensitive to inhibition by NPI-3, nALDH-3 was relatively insensitive to inhibition by NPI-1, and rALDH-1, nALDH-3 and tALDH-3 were relatively insensitive to API-1. In general, the rank order of enzyme sensitivity to these agents was yALDH >> rALDH-2 ≥ tALDH-3 ≥ rALDH-1 > nALDH-3. As judged by IC_{50} values, none of these agents showed much potential as a clinically useful inhibitor of ALDH-3s since none was very potent or specific in that regard. Inhibition by chlorpropamide analogues was relatively specific for substrate-nonspecific aldehyde dehydrogenases since a substrate-specific dehydrogenase, viz., glyceraldehyde-3-phosphate dehydrogenase, and a hydrolase, viz., alkaline phosphatase, were not inhibited by reasonable concentrations of these agents.

"Figure" 2. Inhibition by API-1 of the oxidative reaction catalyzed by yeast and human aldehyde dehydrogenases.

Significant inhibition by chlorpropamide analogues of the hydrolytic reaction catalyzed by aldehyde dehydrogenases was observed only in the cases of yALDH (NPI-3 and API-1) and rALDH-2 (API-1) (Table 2).

"Table" 2. Inhibition by chlorpropamide analogues of yeast and human aldehyde dehydrogenase-catalyzed hydrolysis: IC_{50} values.

Kinetic constants, viz., Km, Vmax and Ki values, defining the catalysis of oxidative reactions by yALDH, rALDH-1, rALDH-2, nALDH-3 and tALDH-3 and inhibition thereof, are given in Tables 3 and 4. Representative plots from which these values were obtained are shown in Figs. 3, 4 and 5. The Km and Vmax values obtained in the present investigation are in good agreement with those reported previously (Dickinson and Haywood, 1987; Lambeir et al., 1991; Dockham et al., 1992; Sreerama and Sladek, 1993, 1994; Sreerama et al., 1995). Irrespective of the chlorpropamide analogue tested, inhibition of yALDH- and rALDH-1-catalyzed oxidation was always noncompetitive (Fig. 3 and data not shown), inhibition of rALDH-2-catalyzed oxidation was always competitive (Fig. 5) or of the mixed type (data not shown), and inhibition of nALDH-3- and tALDH-3-catalyzed oxidation was always competitive (Fig. 4 and data not shown). The general rank order of inhibitor potency as judged by Ki values was identical to that generated on the basis of IC_{50} values, viz., NPI-1 > API-1 > NPI-3. However, the general rank order of enzyme sensitivity to these agents as judged by Ki values, viz., yALDH = rALDH-2 = tALDH-3 > nALDH-3 ≥ rALDH-1, is somewhat different from that generated on the basis of IC_{50} values.

"Table" 3. NAD-linked enzyme-catalyzed oxidation of aldehydes: Km and Vmax values.

"Table" 4. Inhibition by chlorpropamide analogues of yeast and human aldehyde dehydrogenase-catalyzed oxidation: Ki values.

"Figure" 3. Inhibition of rALDH-1-catalyzed oxidation of acetaldehyde by NPI-3: Lineweaver-Burk plot.

"Figure" 4. Inhibition of NAD-linked tALDH-3-catalyzed oxidation of benzaldehyde by API-1: Lineweaver-Burk plot.

"Figure" 5. Inhibition of rALDH-2-catalyzed oxidation of acetaldehyde by NPI-1: Dixon plot.

As judged by Ki values, too, sensitivity of yALDH to inhibition by the three chlorpropamide analogues did not consistently reflect sensitivity of the human aldehyde dehydrogenases to inhibition by them. For example, yALDH and, to a lesser extent, tALDH-3 were sensitive to inhibition by NPI-3, but rALDH-1, rALDH-2 and nALDH-3 were not. Conversely, rALDH-2 was much more sensitive to inhibition by API-1 than was yALDH.

As judged by Ki values, NPI-1 showed some potential as a clinically useful inhibitor of ALDH-3s since it was relatively potent and specific in that regard. Interestingly, but not totally unexpected, tALDH-3, as compared to nALDH-3, was significantly more sensitive to inhibition by each of the three chlorpropamide analogues (Tables 1 and 4). This observation further substantiates the notion that tALDH-3, putatively tumor-specific, is a subtle variant of nALDH-3 (Sladek et al., 1995).

Ki values defining the inhibition of yALDH- and rALDH-2-catalyzed hydrolysis by NPI-3 and API-1 are given in Table 5. Inhibition of yALDH- and rALDH-2-catalyzed hydrolysis by these agents was of the mixed type (data not shown).

"Table" 5. Inhibition by chlorpropamide analogues of yeast and human recombinant class 2 aldehyde dehydrogenase-catalyzed hydrolysis.

A priori, the expectation was that yALDH-, rALDH-1- and rALDH-2-catalyzed oxidation would be much more sensitive to inhibition by NPI-1 and NPI-3 than would be that catalyzed by the two ALDH-3s. This was because 1) as judged by the relative rates at which they catalyze the hydrolysis of *p*-nitrophenyl acetate (see footnote to Table 2), the former were expected to catalyze the bioactivation of the pro-inhibitors at a much faster rate, and 2) the former were much more sensitive to Piloty's acid (Table 1), an agent that spontaneously gives rise to HNO (the inhibitory action of Piloty's acid is thought to be effected by HNO, rather than by the parent compound) (Nagasawa et al., 1995). The expectation was not realized (Tables 1 and 4). Why is uncertain. Hydrolytic release of HNO from NPI-3 catalyzed by yALDH has been demonstrated as has inhibition of yALDH-catalyzed oxidation of acetaldehyde to acetate by HNO, albeit, by deduction (Lee et al., 1992b). It may be that the relative rates at which human aldehyde dehydrogenases catalyze hydrolysis of *p*-nitrophenyl acetate do not reflect the relative rates at which these enzymes catalyze the hydrolysis of NPI-1 and NPI-3. Indeed, NPI-1 and NPI-3 may not even be substrates for human aldehyde dehydrogenases. Even so, HNO may still have been generated given that the pH of the enzyme assay mixture was 8.1 and that, in general, esters of hydroxamic acids readily undergo hydrolysis in aqueous alkaline solutions. NPI-1 and NPI-3 have been shown to undergo rapid ester hydrolysis when placed into a strongly alkaline (0.1 N NaOH) aqueous solution (Lee et al., 1992b). By itself, however, this eventuality, does not explain our findings given that yALDH, rALDH-1 and rALDH-2 are more sensitive to inhibition by HNO (Piloty's acid) than are the ALDH-3s (Table 1). The possibility that the parent compounds themselves effect the inhibitory action is seemingly negated by our preliminary findings that inhibition increased as the time of

preincubation (enzyme and inhibitor) increased from zero to about three min, though not beyond (data not shown).

Several, collectively rather compelling, observations support the notion that, at least in the cases of ALDH-1 and ALDH-2, aldehydes and esters bind to the same amino acid residue, viz., cysteine 302 (Kitson et al., 1991; Blatter et al., 1992; Mukerjee and Pietruszko, 1992, 1994; Farrés et al., 1995). However, some observations are inconsistent with this notion (Klyosov et al., 1996 and the references cited therein). Our findings that the oxidative reactions were inhibited by chlorpropamide analogues whereas the hydrolytic reactions, in general, were not, are, seemingly, also in conflict with the single site hypothesis.

It could be argued that, with the recently made availability of recombinant human aldehyde dehydrogenases, they, rather than yALDH, should be used to screen for aldehyde dehydrogenase inhibitors ultimately expected to be used in humans, especially since the use of yALDH for this purpose can give rise to false positives, e.g., comparing IC_{50} values quantifying NPI-3 inhibition of yALDH (17 μM) and rALDH-2 (900 μM) (Table 1), and, probably less often, false negatives, e.g., comparing Ki values defining API-1 inhibition of yALDH (23 μM) and rALDH-2 (3 μM) (Table 4). In particular, structure-activity relationships generated by this model may not be useful when the goal is to design an optimal inhibitor of one of the human aldehyde dehydrogenases, e.g., comparing Ki values defining the sensitivity of yALDH to NPI-1 (7.2 μM) and NPI-3 (8.3 μM) with the sensitivity of rALDH-2 to NPI-1 (7 μM) and NPI-3 (80 μM) (Table 4). The yALDH model does, however, offer some advantages, viz., yALDH is commercially available, relatively inexpensive, and, in most cases, at least in our limited study, relatively more sensitive to candidate inhibitors.

Perhaps the most economical and rewarding approach would be to use yALDH in primary screening, thereby identifying virtually all candidates that have any possibility of being of value, and then submitting the agents thus identified to secondary screening in which inhibition of the human aldehyde dehydrogenase(s) of interest would be evaluated. Agents showing promise in the secondary screen would then be further evaluated in an appropriate culture, organ and/or whole animal model.

ACKNOWLEDGEMENT

These investigations were supported by Department of Defense Grant DAMD 17-94-J-4057.

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Table 1. Inhibition by chlorpropamide analogues and Piloty's acid of yeast and human aldehyde dehydrogenase-catalyzed oxidation: IC_{50} values[a]

Enzyme	IC_{50}, μM				
	Chlorpropamide	NPI-1	NPI-3	Piloty's acid	API-1
yALDH	> 2,000	13	17	10	24
rALDH-1	> 2,000	49	217	20	150
rALDH-2	> 2,000	47	900	13	23
nALDH-3	> 2,000	121	775	105	223
tALDH-3	> 1,000	45	305	117	93
GAPDH	> 2,000	> 150	303	*b*	> 500
Alkaline phosphatase	> 1,000	> 200	> 500	*b*	> 300

[a]Enzymes were incubated with vehicle or various concentrations of the putative inhibitors for 5 (chlorpropamide, NPI-1, NPI-3 and API-1) or 10 (Piloty's acid) min, substrate was added, and initial catalytic rates were quantified as described in Materials and Methods. Substrates and cofactors were, respectively, acetaldehyde (0.8 mM) and NAD (4 mM) for yALDH, acetaldehyde and NAD (4 mM each) for rALDH-1, acetaldehyde (2 mM) and NAD (4 mM) for rALDH-2, benzaldehyde (4 mM) and NAD (1 mM) for the ALDH-3s, and glyceraldehyde-3-phosphate and NAD (1 mM each) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *p*-Nitrophenyl phosphate (10 mM) was the substrate for alkaline phosphatase. Uninhibited initial catalytic rates (mean ± SE; n = 6) were 9.1 ± 0.4, 0.72 ± 0.02, 1.7 ± 0.1, 29 ± 1, 32 ± 1, 52 ± 2 and 15 ± 1 IU/mg protein for yALDH, rALDH-1, rALDH-2, nALDH-3, tALDH-3, GAPDH and alkaline phosphatase, respectively. Examples of plots of the primary data thus obtained are given in Fig. 2. IC_{50} values were calculated from such data as described in Materials and Methods.

[b]Not determined

Table 2. Inhibition by chlorpropamide analogues of yeast and human aldehyde dehydrogenase-catalyzed hydrolysis: IC_{50} values[a]

Enzyme	IC_{50}, μM			
	Chlorpropamide	NPI-1	NPI-3	API-1
yALDH	> 2,000	> 100	40	7.3
rALDH-1	> 2,000	> 100	> 1,000	> 400
rALDH-2	> 2,000	> 100	> 1,000	19
nALDH-3	> 2,000	> 100	388	> 400
tALDH-3	> 2,000	> 100	> 1,000	> 400

[a]Enzymes were incubated with vehicle or various concentrations of the putative inhibitors for 5 min, substrate (500 μM *p*-nitrophenyl acetate) was added, and initial catalytic rates were quantified as described in Materials and Methods. Uninhibited initial catalytic rates (mean ± SE; n = 6) were 427 ± 9, 155 ± 6, 583 ± 15, 8.7 ± 0.2 and 8.7 ± 0.6 IU/mg protein for yALDH, rALDH-1, rALDH-2, nALDH-3 and tALDH-3, respectively.

Table 3. NAD-linked enzyme-catalyzed oxidation of aldehydes: Km and Vmax values[a]

Enzyme	Substrate	NAD, mM	n	Km ± SE, μM	Vmax ± SE, IU/mg
yALDH	Acetaldehyde	4	4	34 ± 3	10 ± 1
rALDH-1	Acetaldehyde	4	4	429 ± 61	0.64 ± 0.02
rALDH-2	Acetaldehyde	4	1	4[b]	1.9 ± 0.0
nALDH-3	Benzaldehyde	1	4	414 ± 14	30 ± 1
tALDH-3	Benzaldehyde	1	4	357 ± 12	32 ± 1

[a]Initial catalytic rates were quantified, and Km and Vmax values were calculated, as described in Materials and Methods. Examples of plots of primary data from which the Km and Vmax, as well as Ki (Table 4), values were estimated are given in Figs. 3 and 4.

[b]Unlikely to be accurate because it is difficult to ascertain Km values that are less than about 10 μM from the very flat Lineweaver-Burk plots that we generated. Thus, the Km value was determined to be < 0.1 μM when a more appropriate experimental design and method of analysis, viz., integrated Michaelis analysis of a single enzyme-progress curve, was used (Dockham et al., 1992).

Table 4. Inhibition by chlorpropamide analogues of yeast and human aldehyde dehydrogenase-catalyzed oxidation: Ki values[a]

Enzyme	Ki, μM		
	NPI-1	NPI-3	API-1
yALDH	7.2	8.3	23
rALDH-1	21	142	138
rALDH-2	7.0	80	3.0
nALDH-3	18	153	34
tALDH-3	3.9	26	19

[a]Enzymes were incubated with vehicle or various concentrations of the putative inhibitors for 5 min, substrate was added, and initial catalytic rates were quantified as described in Materials and Methods. Cofactor concentrations and substrates were as listed in Table 3. Km and Vmax values defining each of the enzyme-catalyzed reactions are also given in Table 3. Examples of plots of primary data from which the Ki values were estimated are given in Figs. 3, 4 and 5.

Table 5. Inhibition by chlorpropamide analogues of yeast and human recombinant class 2 aldehyde dehydrogenase-catalyzed hydrolysis[a]

Enzyme	Ki, μM		
	NPI-1	NPI-3	API-1
yALDH	[b]	73	3.5
rALDH-2	[b]	[b]	22

[a]Enzymes were incubated with vehicle or various concentrations of the putative inhibitors for 5 min, the substrate, ~~500 μM~~ *p*-nitrophenyl acetate, was added, and initial catalytic rates were quantified as described in Materials and Methods. Km and Vmax values (mean ± SE) for the yALDH-catalyzed reaction (n = 3) were 37 ± 6 μM and 342 ± 55 IU/mg, respectively. Km and Vmax values for the rALDH-2-catalyzed reaction (n = 1) were 30 μM and 584 IU/mg, respectively.

[b]Ki values were not determined because inhibition was less than 20% at the highest concentration of inhibitor tested, Table 2.

FIGURE LEGENDS

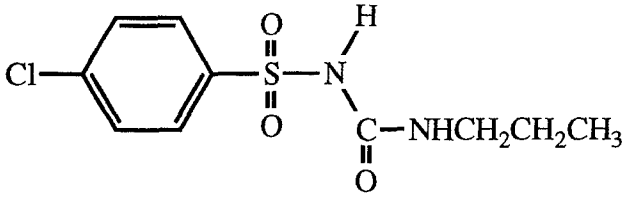
Figure 1. Structures of chlorpropamide, three analogues thereof, and Piloty's acid.

Figure 2. Inhibition by API-1 of the oxidative reaction catalyzed by yeast and human aldehyde dehydrogenases. The sensitivities of yALDH (●), rALDH-1 (○), rALDH-2 (▲), nALDH-3 (△) and tALDH-3 (□) to inhibition by API-1 were determined as described in Materials and Methods and a footnote to Table 1. Data points are means of duplicate determinations. Control catalytic rates are given in a footnote to Table 1. Best-fit curves, and IC_{50} values estimated therefrom, were generated from the data presented in this figure as described in Materials and Methods. IC_{50} values thus obtained are given in Table 1.

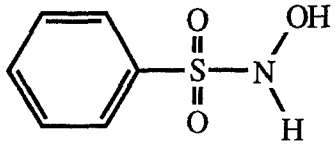
Figure 3. Inhibition of rALDH-1-catalyzed oxidation of acetaldehyde by NPI-3: Lineweaver-Burk plot. rALDH-1 was incubated with 0 (○), 90 (●), 180 (□) or 360 (■) μM NPI-3 for 5 min, various concentrations of the substrate, acetaldehyde, were added, and initial catalytic rates were quantified as described in Materials and Methods. The NAD concentration was 4 mM. Data points are means of triplicate determinations. Inset: Slopes generated by the double-reciprocal (Lineweaver-Burk) plots were plotted as a function of NPI-3 concentrations for the purpose of determining the Ki value. Km, Vmax and Ki values obtained in this experiment were 434 μM, 0.66 IU/mg and 142 μM, respectively.

Figure 4. Inhibition of NAD-linked tALDH-3-catalyzed oxidation of benzaldehyde by API-1: Lineweaver-Burk plot. tALDH-3 was incubated with 0 (○), 25 (●), 50 (□) or 75 (■) μM API-1 for 5 min, various concentrations of the substrate, benzaldehyde, were added, and initial catalytic rates were quantified as described in Materials and Methods. The NAD concentration was 1 mM. Data points are means of triplicate determinations. Inset: Slopes generated by the double-reciprocal (Lineweaver-Burk) plots were plotted as a function of API-1 concentrations for the purpose of determining the Ki value. Km, Vmax and Ki values obtained in this experiment were 337 μM, 30 IU/mg and 19 μM, respectively.

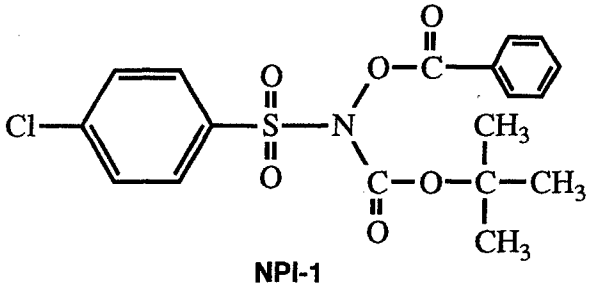
Figure 5. Inhibition of rALDH-2-catalyzed oxidation of acetaldehyde by NPI-1: Dixon plot. rALDH-2 was incubated with vehicle or various concentrations of NPI-1 for 5 min, 250 (○), 500 (●) or 1000 (□) μM acetaldehyde was added, and initial catalytic rates were determined as described in Materials and Methods. The NAD concentration was 4 mM. Data points are means of triplicate determinations. The Ki value was 7 μM. ■, Vmax determined by a Lineweaver-Burk plot (not shown) was 1.9 IU/mg. The Km as determined by a Lineweaver-Burk plot was 3.6 μM.



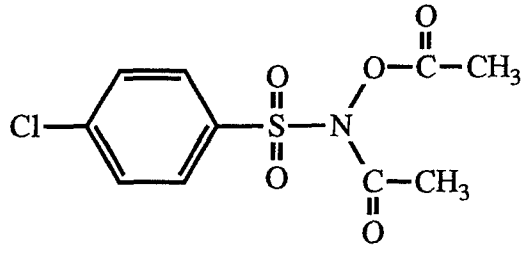
Chlorpropamide



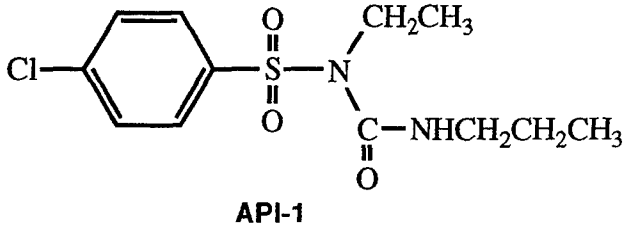
Piloty's acid



NPI-1



NPI-3



API-1

Chlorpropamide: 1-(p-chlorobenzenesulfonyl)-3-*n*-propylurea

Piloty's acid: benzenesulfohydroxamic acid

NPI-1: (benzoyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester

NPI-3: N-acetyl-N-(acetyloxy)-4-chlorobenzenesulfonamide

API-1: 4-chloro-N-ethyl-N-[(propylamino)carbonyl]benzenesulfonamide

Figure 1.

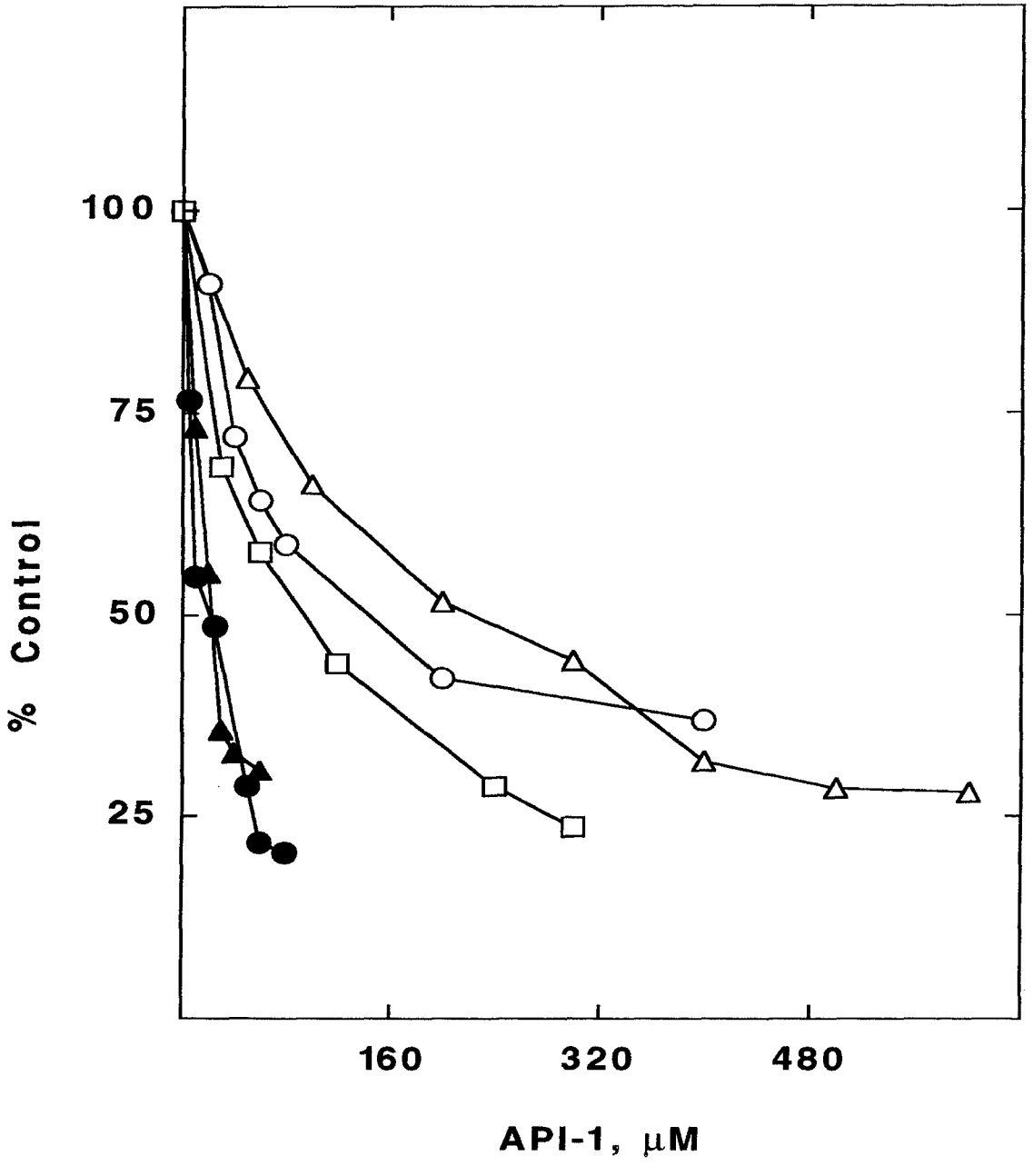


Figure 2.

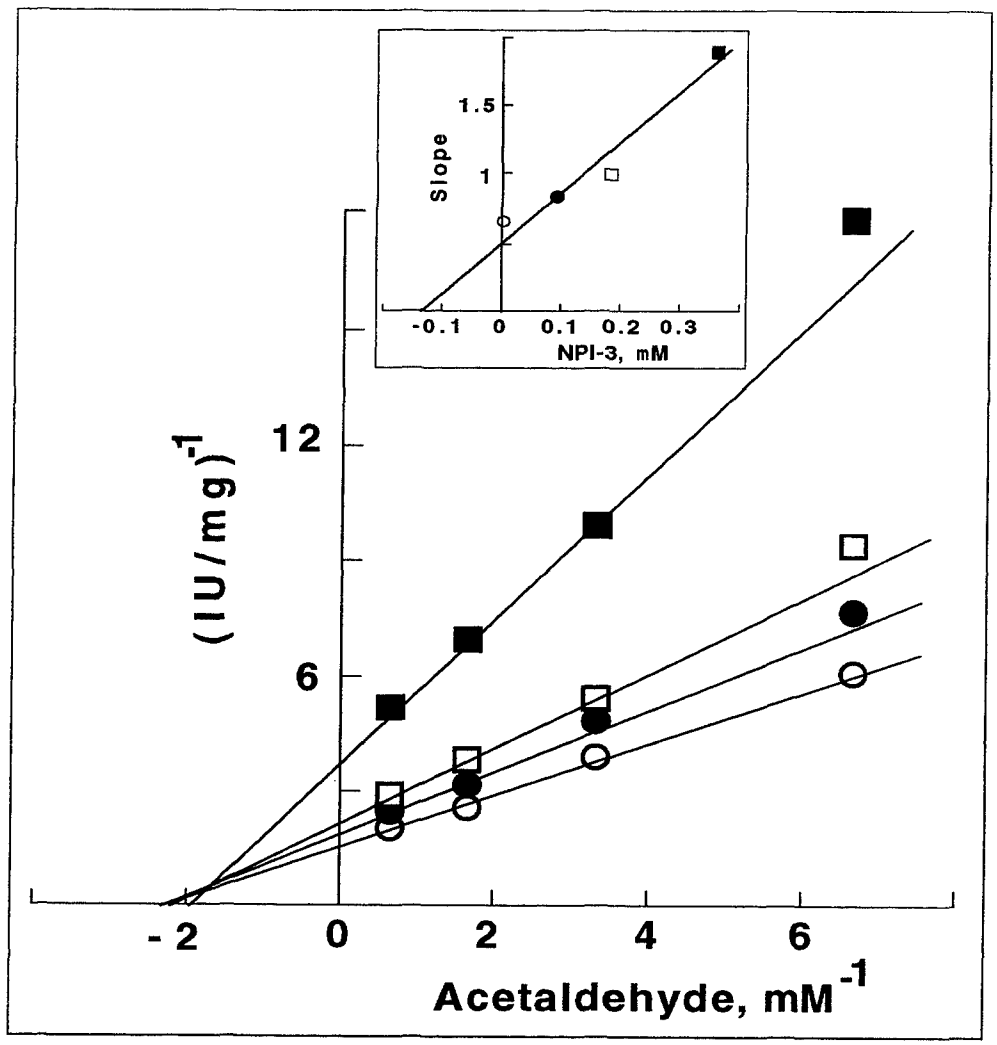

Slope
1.5
1
-0.1
0
0.1
0.2
0.3
NPI-3, mM
(IU/mg)-1
12
6
-2
0
2
4
6
Acetaldehyde, mM-1

Figure 3.

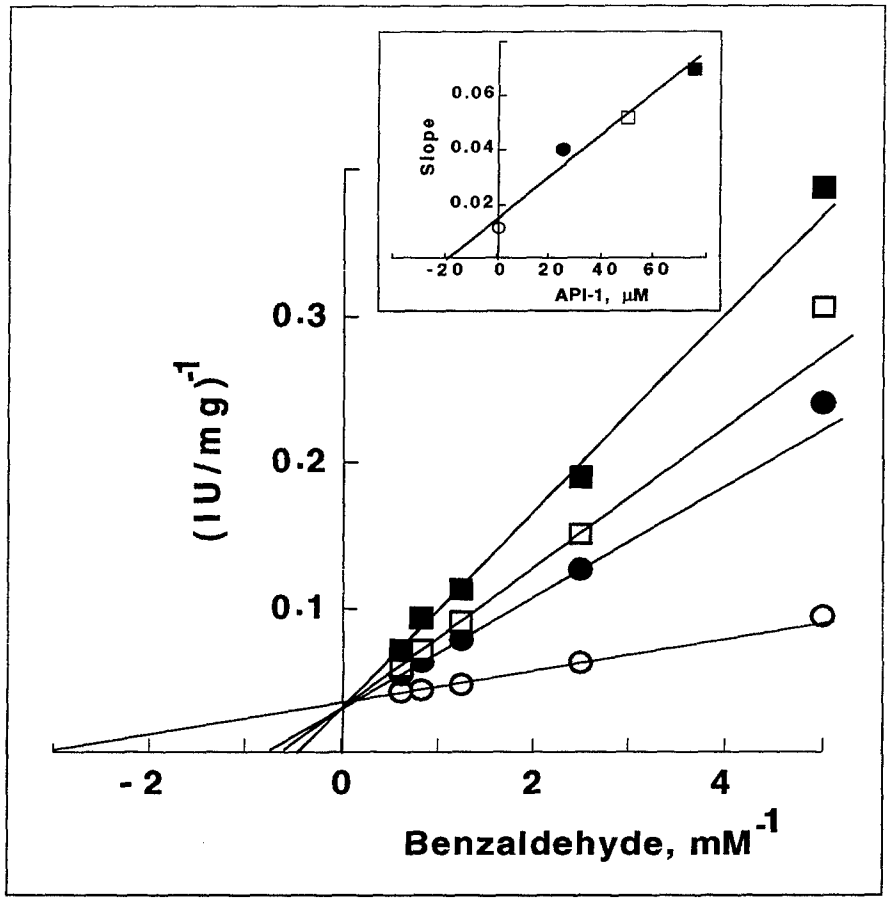

Slope
0.06
0.04
0.02
-20
0
20
40
60
API-1, µM
0.3
0.2
0.1
(IU/mg)$^{-1}$
-2
0
2
4
Benzaldehyde, mM^{-1}

Figure 4.

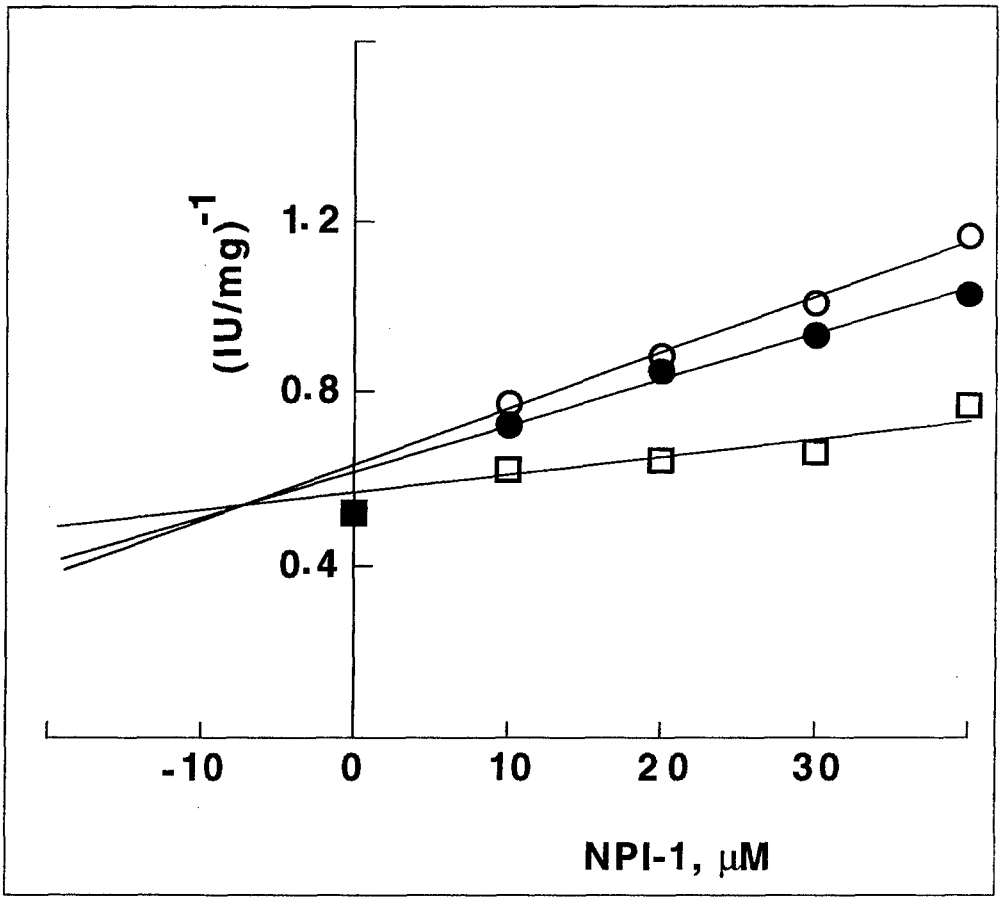


Figure 5.

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Weiner et al. (eds.), 1997, In press.

INHIBITION OF HUMAN CLASS 3 ALDEHYDE DEHYDROGENASE, AND SENSITIZATION OF TUMOR CELLS THAT EXPRESS SIGNIFICANT AMOUNTS OF THIS ENZYME TO OXAZAPHOSPHORINES, BY THE NATURALLY OCCURRING COMPOUND GOSSYPOL

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INTRODUCTION

Cytosolic class 3 aldehyde dehydrogenase (ALDH-3) is a demonstrated determinant of cellular sensitivity to the cytotoxic action of certain widely used antineoplastic prodrugs collectively referred to as oxazaphosphorines, e.g., cyclophosphamide, ifosfamide, 4-hydroperoxycyclophosphamide, 4-hydroperoxyifosfamide and mafosfamide (cellular sensitivity to these drugs decreases as cellular levels of ALDH-3 increase) (Sladek, 1993; Sreerama and Sladek, 1993a,b, 1994; Bunting et al., 1994; Rekha et al., 1994; Sladek et al., 1995; Sreerama et al., 1995; Bunting and Townsend, 1996). Thus, tumor cells, otherwise sensitive to the oxazaphosphorines, became resistant to these drugs when electroporated with purified ALDH-3 protein or transfected with the cDNA coding for ALDH-3 (Bunting et al., 1994; Sreerama and Sladek, 1995; Bunting and Townsend, 1996), and, of therapeutic significance, relatively elevated levels of this enzyme can account for intrinsic, transient acquired, and stable acquired, resistance to the oxazaphosphorines on the part of malignant cells (Sreerama and Sladek, 1993a,b, 1994; Rekha et al., 1994; Sreerama et al., 1995). Resistance to the oxazaphosphorines mediated by ALDH-3 is ostensibly due to the enzyme-catalyzed oxidative detoxification of aldophosphamide, the pivotal metabolite of these prodrugs (Sreerama and Sladek, 1993a, 1994; Rekha et al., 1994; Sreerama et al., 1995; Bunting and Townsend, 1996).

Inhibition of ALDH-3 would therefore be expected to sensitize otherwise relatively insensitive tumor cells to the oxazaphosphorines when relatively high cellular levels of ALDH-3 is the basis for the relative insensitivity. Thus, inhibitors of ALDH-3 could be of therapeutic value. However, to date, no inhibitor of ALDH-3 has been identified, although inhibition of ALDH-3-catalyzed oxidation of aldophosphamide to carboxyphosphamide by alternative substrates can be effected, *vide infra*. Known inhibitors of class 1 (ALDH-1) and/or class 2 (ALDH-2) aldehyde dehydrogenases, e.g., disulfiram and chloral hydrate, do not, or only minimally, inhibit ALDH-3 (Sreerama and Sladek, 1993a, 1994) and, predictably, do not sensitize tumor cells to the oxazaphosphorines when such cells are insensitive to these agents because of relatively high ALDH-3 levels (reviewed in Sladek et al., 1995). Another inhibitor of ALDH-1, viz., 4-(diethylamino)benzaldehyde, proved to be a substrate for ALDH-3 (Rekha et al., 1994; Sladek et al., 1995). Alternative substrates for ALDH-3, e.g., benzaldehyde and 4-(diethylamino)benzaldehyde, compete with oxazaphosphorines for the catalytic site and thus sensitize tumor cells that express relatively large amounts of ALDH-3 and are therefore otherwise relatively insensitive to these agents (Sreerama and Sladek, 1994; Rekha et al., 1994).

In our search for an inhibitor of ALDH-3, gossypol, a polyphenolic aldehyde (Fig. 1) found in cottonseed extracts, emerged as a possible candidate. It is known to inhibit several NAD(P)-linked dehydrogenases, e.g, lactate dehydrogenase (Lee et al., 1982; Olgiati and Toscano, 1983; Burgos et al., 1986), malate dehydrogenase (Burgos et al., 1986), glutamate dehydrogenase (Burgos et al., 1986), glyceraldehyde-3-phosphate dehydrogenase (Ikeda, 1990), 11-β-hydroxysteroid dehydrogenase (Sang et al., 1991) and alcohol dehydrogenase (Messiha, 1991a). Directly germane to our interests, gossypol has

been shown to inhibit "hepatic aldehyde dehydrogenase" activity when given to mice (Messiha, 1991a,b). Therefore, we initiated studies intended to ascertain the effect of gossypol on the catalytic activity of ALDH-3.

"Figure" 1. Structure of gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-8,8'-dicarboxyaldehyde].

ALDH-1, known to also catalyze the irreversible oxidation (detoxification) of aldophosphamide (Dockham et al., 1992), and ALDH-2 were included in our investigations so that the relative specificity, if any, of the inhibitory effect of gossypol towards each of the three classes of aldehyde dehydrogenases could be ascertained.

The ALDH-3 present in human tumor cells/tissues (tALDH-3), e.g., cultured breast adenocarcinoma MCF-7 cells, colon carcinoma C cells, and salivary gland Warthin tumors and mucoepidermoid carcinomas, although otherwise seemingly identical to the ALDH-3 present in human normal tissues/fluids (nALDH-3), e.g., stomach mucosa and saliva, differs from the latter in that it exhibits a much greater ability to catalyze the oxidative detoxification of the oxazaphosphorines (reviewed in Sladek et al., 1995). Hence, both tALDH-3 and nALDH-3 were included in our investigations.

Aldehyde dehydrogenases are bifunctional enzymes, i.e., they catalyze both oxidative (oxidation of aldehydes to acids) and hydrolytic (hydrolysis of esters) reactions. Thus, the effect of gossypol on each of these reactions was determined.

Gossypol has been shown to be toxic to various human tumor cells including human breast adenocarcinoma MCF-7 cells (Tuszynski and Cossu, 1984; Joseph et al., 1986; Band et al., 1989; Wu et al., 1989; Benz et al., 1990; Ford et al., 1991; Hu et al., 1993; Coyle et al., 1994; Gilbert et al., 1995) and is being tested clinically for its potential as an anticancer agent (Wu, 1989; Stein et al., 1992; Flack et al., 1993; Seidman, 1996). The sensitivity of cultured human adenocarcinoma MCF-7/0 (cellular levels of ALDH-3 are very low) and MCF-7/0/CAT (cellular levels of ALDH-3 are relatively high) cells to gossypol was determined in the present investigation. The ability of gossypol to negate the influence of relatively high cellular levels of ALDH-3 on the cellular sensitivity of tumor cells to oxazaphosphorines was also determined.

MATERIALS AND METHODS

Mafosfamide was provided by Dr. J. Pohl, Asta Medica AG, Frankfurt, Germany. Phosphoramide mustard·cyclohexylamine was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. *E. coli* [BL21(DE3)pLysS] transfected with pET-19b vector to which human ALDH-1 cDNA [cloned from human hepatoma Hep G2 cells (Moreb et al., 1996)] was ligated, was provided by Dr. Jan Moreb, University of Florida, Gainsville, FL. A vector, viz., pT7-7, to which human ALDH-2 cDNA [cloned from human liver (Zheng et al., 1993)] was ligated, was provided by Dr. Henry Weiner, Purdue University, Lafayette, IN. Transfection of human ALDH-2 cDNA ligated to the pT7-7 vector into *E. coli* [BL21(DE3)pLysS] was by Drs. P. A. Dockham and L. Sreerama of our laboratory as described by Sambrook et al. (1989). Generation and purification of recombinant human ALDH-1 (rALDH-1) and ALDH-2 (rALDH-2) were by Dr. V. R. Devaraj of our laboratory [for details see Devaraj, V.R., Sreerama, L., Lee, M.J.C., Nagasawa, H.T. and Sladek, N.E.: Chlorpropamide analogs as inhibitors of human aldehyde dehydrogenases. In Weiner, H., Lindahl, R., Crabb, D.W. and Flynn, T.G. (Eds.), Enzymology and Molecular Biology of Carbonyl Metabolism - 6, Plenum Press, New York, 1997]. Human erythrocyte glyceraldehyde-3-phosphate dehydrogenase, human placental alkaline

phosphatase type XXIV, racemic gossypol and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals and reagents were obtained from the sources listed in previous publications (Dockham et al., 1992; Sreerama and Sladek, 1993a, 1994; Sreerama et al., 1995).

Human normal stomach mucosa ALDH-3 (nALDH-3) and the ALDH-3 (tALDH-3) present in human breast adenocarcinoma MCF-7/0 cells cultured in the presence of 30 μM catechol for 5 days to induce the enzyme (MCF-7/0/CAT cells) were purified as described previously (Sreerama and Sladek, 1993b; Sreerama et al., 1995).

NAD-linked oxidation of acetaldehyde catalyzed by rALDH-1 and rALDH-2 at 37°C and pH 8.1, NAD(P)-linked oxidation of benzaldehyde catalyzed by nALDH-3 and tALDH-3 at 37°C and pH 8.1, and hydrolysis of *p*-nitrophenyl acetate catalyzed by each of these enzymes at 25°C and pH 7.5, were quantified spectrophotometrically as described previously (Dockham et al., 1992; Sreerama and Sladek, 1993a). NAD-linked oxidation of glyceraldehyde-3-phosphate catalyzed by glyceraldehyde-3-phosphate dehydrogenase at 37°C and pH 7.6, and hydrolysis of *p*-nitrophenyl phosphate catalyzed by alkaline phosphatase at 25°C and pH 9.8, were quantified spectrophotometrically as described by Lambeir et al. (1991) and Chueh et al. (1981), respectively. Preincubation of gossypol or vehicle together with the complete reaction mixture except for the substrate was for 5 min. Preliminary experiments revealed that the degree of gossypol-mediated inhibition of ALDH-catalyzed oxidation did not increase when preincubation of gossypol with the enzyme was for 20, rather than 5, min. Preincubation temperatures and pHs were the same as incubation temperatures and pHs. All reactions were started by the addition of substrate. Stock ethanol solutions of gossypol were prepared freshly each day and were stored protected from light, on ice, before addition to the reaction mixture. The final concentration of ethanol in the reaction mixture was always 0.5%; this concentration of ethanol did not inhibit any of the enzyme-catalyzed reactions under investigation.

Human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells were cultured (monolayer), harvested when still in exponential growth, resuspended in growth medium, and checked for viability (usually greater than 95% as judged by trypan blue exclusion) as described previously (Sreerama and Sladek, 1993a; Sreerama et al., 1995). Drug exposure and the colony-forming assay used to determine surviving fractions were also as described previously (Sreerama and Sladek, 1993a). Briefly, freshly harvested cells were diluted with drug-exposure medium to a concentration of 1×10^5 cells/ml and then exposed to drug (mafosfamide or phosphoramide mustard) or vehicle for 30 min at pH 7.4 and 37°C after which they were harvested and cultured in drug-free growth medium for 15 days. Colonies (≥ 50 cells) were then visualized with methylene blue dye and counted. Stock solutions of mafosfamide and phosphoramide mustard were prepared by placing them into aqueous solution just before use. In some experiments, cells were preincubated with 75 μM gossypol or vehicle for 5 min at 37°C prior to the addition of mafosfamide or phosphoramide mustard. Stock ethanol solutions of gossypol were diluted with drug-exposure medium just before use. Ethanol concentrations in the drug-exposure medium never exceeded 0.5%; this concentration of ethanol did not affect the rate of cell proliferation. At the concentration used (75 μM), gossypol effected only a small amount of cell-kill (< 10%) and this was taken into account when calculating the effect of including gossypol in the drug-exposure medium on LC_{90} (concentration of drug required to effect 90% cell-kill) values for mafosfamide and phosphoramide mustard.

Double-reciprocal (Lineweaver-Burk) plots of initial catalytic rates (determined in duplicate) as a function of substrate concentrations (at least four and usually six) were used to estimate the Km and Vmax values. Ki values were determined by plotting the slopes of the lines generated by the double-reciprocal (Lineweaver-Burk) plots as a function of gossypol

concentrations. In the case of double-reciprocal (Lineweaver-Burk) plots, computer-assisted Wilkinson weighted linear regression analysis (Wilkinson, 1961) effected by the MacWilkins program (Microsoft, Bellevue, WA) was used to generate best-fit lines. Computer-assisted unweighted linear regression analysis effected by the STATView statistical program (Brainpower Inc., Calabas, CA) was used to generate best-fit lines for all other straight-line functions.

RESULTS AND DISCUSSION

Gossypol was not a substrate for either the oxidative (oxidation of aldehydes) or the hydrolytic (hydrolysis of esters) reactions catalyzed by any of the ALDHs studied.

Oxidative reactions catalyzed by rALDH-1, rALDH-2 and the ALDH-3s were inhibited by gossypol (Fig. 2 and Table 1). As judged by the concentrations of gossypol required to effect 50% inhibition (IC_{50}), ALDH-3s were, relative to rALDH-1, rALDH-2 and human glyceraldehyde-3-phosphate dehydrogenase, far more sensitive to inhibition by gossypol. Inhibition could not be reversed by passing the enzyme·inhibitor complex (tALDH-3·gossypol generated by incubating tALDH-3 with an amount of gossypol that inhibited tALDH-3-catalyzed oxidation of benzaldehyde by 85%, viz., 20 μM, for 5 min) through a PD-10 (Sephadex G-25) column (data not shown). Seemingly inconsistent with our findings, others have reported that inhibition of lactate dehydrogenase-X by gossypol was reversed when the enzyme·inhibitor complex was passed through a Bio Gel P4 column (Olgiati and Toscano, 1983).

"Figure" 2. Inhibition by gossypol of oxidative reactions catalyzed by human aldehyde dehydrogenases.

"Table" 1. Inhibition by gossypol of human aldehyde dehydrogenase-catalyzed oxidation and hydrolysis: IC_{50} values.

Fifty-percent inhibition of a hydrolytic reaction (hydrolysis of *p*-nitrophenyl acetate) catalyzed by the ALDHs was not achieved at the highest concentration of gossypol, 200 μM, tested (Table 1). As expected (Feldman and Weiner, 1972; Sidhu and Blair, 1975; Sreerama and Sladek, 1993a), inclusion of 1 mM NAD in the reaction mixture increased the rates at which ALDH-1 and ALDH-2 catalyzed the hydrolysis of *p*-nitrophenyl acetate (3- and 6-fold, respectively), but not the rates at which the ALDH-3s did so (data not shown). As when NAD was not included in the incubation mixture, 50% inhibition of ALDH-catalyzed hydrolysis by gossypol, 200 μM, was not achieved when it was (data not shown). The hydrolytic reaction (hydrolysis of *p*-nitrophenyl phosphate) catalyzed by human placental AP was inhibited by gossypol, albeit poorly (Table 1).

As judged by Ki values determined with respect to the substrate, as well as those determined with respect to the cofactor, ALDH-3-catalyzed oxidation was inhibited to a greater extent by gossypol than was that catalyzed by rALDH-1 and rALDH-2 (Table 2). Inhibition was always noncompetitive with respect to the aldehyde. A double-reciprocal (Lineweaver-Burk) plot illustrating this point in the case of tALDH-3 is shown in Fig. 3. Inhibition was always competitive with respect to the cofactor. A double-reciprocal (Lineweaver-Burk) plot illustrating this point in the case of tALDH-3 is shown in Fig. 4. Thus, gossypol competes with the cofactor, rather than with the aldehyde, for a binding site.

"Table" 2. Inhibition by gossypol of human aldehyde dehydrogenase-catalyzed oxidation: Ki values.

"Figure" 3. Inhibition of NAD-linked tALDH-3-catalyzed oxidation of benzaldehyde by gossypol: Lineweaver-Burk plot of initial catalytic rates as a function of substrate, benzaldehyde, concentrations.

"Figure" 4. Inhibition of NAD-linked tALDH-3-catalyzed oxidation of benzaldehyde by gossypol: Lineweaver-Burk plot of initial catalytic rates as a function of cofactor, NAD, concentrations.

Gossypol has been shown to inhibit a variety of NAD(P)-linked enzyme-catalyzed oxidations, *vide supra*, as well as NAD(P)H-linked enzyme-catalyzed reductions, e.g., those catalyzed by aldose reductase (Deck et al., 1991) and 5α-reductase (Moh et al., 1993). Consistent with our findings, inhibition was, with two exceptions, noncompetitive with respect to the substrate and competitive with respect to the pyridine nucleotide in each of the cases where this determination was made (Burgos et al., 1986; Ikeda, 1990; Moh et al., 1993). One of the exceptions was lactate dehydrogenase-X-catalyzed reduction of α-ketobutyrate where inhibition was competitive with respect to the substrate and noncompetitive with respect to the pyridine nucleotide (Morris et al., 1986). The other was 11-β-hydroxysteroid dehydrogenase-catalyzed oxidation of corticosterone where inhibition was competitive with respect to the substrate (Sang et al., 1991).

Gossypol has also been shown to inhibit enzymes that are not dehydrogenases or reductases, e.g., glutathione S-transferases (Lee et al., 1982; Benz et al., 1990), DNA polymerase α (Rosenberg et al., 1986), protein kinase C (Nakadate et al., 1988; Benz et al., 1990), calmodulin-stimulated cyclic AMP phosphodiesterase (Benz et al., 1990), and adenylate cyclase (Olgiati et al., 1984). Interestingly, inhibition of adenylate cyclase by gossypol was competitive and it has been inferred that gossypol may inhibit all enzymes for which pyridine nucleotides or ATP are a substrate or cofactor (Olgiati and Toscano, 1983; Olgiati et al., 1984).

Initially, gossypol was of investigative interest mainly because of its male contraceptive properties (reviewed in Wu, 1989). Subsequently, its therapeutic potential in the treatment of certain gynecological diseases, e.g., endometriosis and uterine myoma, became of interest (reviewed in Wu, 1989). Recognition, during the course of those investigations, of its inhibitory effect on a number of intracellular enzymes important for cellular growth led to considerable interest in developing other therapeutic uses for gossypol, viz., the chemotherapy of microbial, parasitic and neoplastic diseases. Thus, it was soon demonstrated that gossypol and its derivatives inhibit the *in vitro* replication of certain viruses (Wichmann, et al., 1982; Royer, et al., 1991), *in vitro* growth of *Trypanosoma cruzi*, the parasite that causes Chagas' disease (Montamat et al., 1982), and, most notably, the *in vitro* proliferation of a variety of human tumor cell lines, e.g., those derived from breast, hepatic, pancreatic, ovarian, testicular, colon, skin, adrenal, cervical and brain carcinomas (Tuszynski and Cossu, 1984; Joseph et al., 1986; Band et al., 1989; Wu et al., 1989; Benz et al., 1990; Ford et al., 1991; Hu et al., 1993; Coyle et al., 1994; Gilbert et al., 1995). Moreover, gossypol potently inhibited proliferation of doxorubicin-resistant (Benz et al., 1990; Hu et al., 1993), as well as estrogen-responsive and estrogen-nonresponsive (Gilbert et al., 1995), human breast cancer cells. Most importantly, it was demonstrated in several animal models that tumor cell-kill could be achieved *in vivo* with doses of gossypol that were not injurious to the host animal (Tso, 1984; Wu et al., 1989; Chang et al., 1993; Naik et al., 1995). These observations prompted clinical trials of gossypol for the treatment of certain cancers, viz., metastatic carcinomas of the ovary (Wu, 1989), various advanced cancers (Stein et al., 1992), metastatic adrenocortical carcinomas (Flack et al., 1993) and metastatic breast cancers (Seidman, 1996).

In our investigations, gossypol was toxic to human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells (Fig. 5). LC_{90} values were 175 and 205 μM, respectively. Proliferation of MCF-7/0 and MCF-7/0/CAT cells was essentially unaffected by 75 μM gossypol.

"Figure" 5. Sensitivity of human breast adenocarcinoma MCF-7/0 cells that had been grown in the presence and absence of catechol to gossypol.

Addition of gossypol (75 μM) to the drug-exposure medium prior to exposure to mafosfamide markedly increased the sensitivity of tumor cells that express large amounts of ALDH-3, viz., MCF-7/0/CAT, to the oxazaphosphorine (Fig. 6 and Table 3). As expected, identical treatment of tumor cells that express very small amounts of ALDH-3, viz., MCF-7/0, only very minimally increased their sensitivity to mafosfamide. Also as expected because ALDH-3 does not catalyze the detoxification of phosphoramide mustard, the ultimate cytotoxic metabolite of mafosfamide (Sladek, 1994), addition of gossypol to the drug-exposure medium prior to exposure to this agent essentially did not increase the sensitivity of MCF-7/0/CAT cells to it (Table 3).

"Figure" 6. Sensitivities of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide in the presence and absence of gossypol.

"Table" 3. Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide and phosphoramide mustard in the presence and absence of gossypol.

Racemic gossypol was used in our, as well as in most other, investigations, including the clinical trials referred to above. Significant differences in the potency of the gossypol enantiomers with respect to inhibition of proliferation, fertilization and enzyme catalysis have been reported. Thus, the (−)enantiomer has been shown to be more toxic to cultured tumor cells than was the (+)enantiomer or the racemate (Joseph et al., 1986; Band et al., 1989; Benz et al., 1990; Ford et al., 1991), and some researchers have attributed the contraceptive properties of gossypol entirely to the (−)enantiomer, although others have reported that the two enantiomers are almost equipotent in that regard (reviewed in Yu, 1987). Some enzymes, viz., glutathione S-transferases α and π, have been shown to be inhibited to a greater extent by the (−)enantiomer (Benz et al., 1990), but others, viz., protein kinase C, calmodulin-stimulated cyclic AMP phosphodiesterase, DNA polymerase α and glutathione S-transferase μ, are reportedly each almost equally sensitive to inhibition by the two enantiomers (Rosenberg et al., 1986; Benz et al., 1990), and equisensitivity to the enantiomers on the part of rat and human lactate dehydrogenase-X (Kim et al., 1985; Morris et al., 1986; Yao et al., 1987), as well as greater sensitivity to the (−)enantiomer on the part of the hamster orthologue (Morris et al., 1986; Den Boer and Grootegoed, 1988), have been reported. The relative ability of the two enantiomers to inhibit ALDH-catalyzed oxidations remains to be determined. Seemingly relevant to any potential clinical use of gossypol, marked differences in the disposition of the two enantiomers have been documented (reviewed in Yu, 1987). For example, the plasma half-life of the (−)enantiomer is 4.55 hours whereas that of the (+)enantiomer is 133 hours in patients receiving a single oral dose of racemic gossypol (Wu et al., 1986).

Gossypol is found in the leaves, roots and seeds of the cotton plant. Commercial products originating from these plants include cottonseed meal, used extensively as a protein supplement in animal feed, and cottonseed oil, frequently used for processing human food. Federal (United States) regulation limits the concentration of free gossypol in animal feed, as well as in products intended for human consumption, to 450 ppm (FDA, 1974). Gossypol has been found to be present in milk collected from cows that had been fed the federally allowable 450 ppm gossypol for six days (Hu et al., 1994). Uncertain is whether

dietary intake of gossypol by humans is sufficient to inhibit aldehyde dehydrogenases, and thus the rate at which oxazaphosphorines, ethanol (acetaldehyde), etc., are detoxified, but that would seem unlikely, at least in the United States.

In any event, the findings reported herein establish the therapeutic potential of combining gossypol with an oxazaphosphorine in the treatment of certain cancers. Moreover, given the antitumor activity that gossypol itself exhibits, it can be envisaged that in the case of some of these cancers, gossypol could be of dual therapeutic value when combined with an oxazaphosphorine in the therapeutic protocol.

ACKNOWLEDGEMENT

These investigations were supported by USPHS Grant CA 21737 and Department of Defense Grant DAMD 17-94-J-4057.

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FIGURE LEGENDS

Figure 1. Structure of gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-8,8'-dicarboxyaldehyde].

Figure 2. Inhibition by gossypol of oxidative reactions catalyzed by human aldehyde dehydrogenases. The sensitivities of rALDH-1 (O), rALDH-2 (▲), nALDH-3 (Δ), and tALDH-3 (□) to inhibition by gossypol were determined as described in Materials and Methods and the footnotes to Table 1. Data points are means of duplicate determinations. Control catalytic rates are listed in a footnote to Table 1. IC_{50} values were estimated directly from the plots shown in this figure and are given in Table 1.

Figure 3. Inhibition of NAD-linked tALDH-3-catalyzed oxidation of benzaldehyde by gossypol: Lineweaver-Burk plot of initial catalytic rates as a function of substrate, benzaldehyde, concentrations. tALDH-3 was incubated with 0 (O), 5 (●), 7.5 (Δ) or 10 (▲) μM gossypol for 5 min, various concentrations of the substrate, benzaldehyde, were added, and initial catalytic rates were quantified as described in Materials and Methods. The NAD concentration was 1 mM. Data points are means of duplicate determinations. Inset: Slopes generated by double-reciprocal (Lineweaver-Burk) plots were plotted as a function of gossypol concentrations for the purpose of determining the Ki value. Km, Vmax and Ki values obtained in this experiment were 349 μM, 35 IU/mg and 4.3 μM, respectively.

Figure 4. Inhibition of NAD-linked tALDH-3-catalyzed oxidation of benzaldehyde by gossypol: Lineweaver-Burk plot of initial catalytic rates as a function of cofactor, NAD, concentrations. tALDH-3 was incubated with 0 (O), 2 (●), 4 (Δ) or 7.5 (▲) μM gossypol for 5 min, the substrate, benzaldehyde (4 mM), was added, and initial catalytic rates were quantified as described in Materials and Methods. Data points are means of duplicate determinations. Inset: Slopes generated by the double-reciprocal (Lineweaver-Burk) plots were plotted as a function of gossypol concentrations for the purpose of determining the Ki value. Km, Vmax and Ki values obtained in this experiment were 47 μM, 32 IU/mg and 0.10 μM, respectively.

Figure 5. Sensitivity of human breast adenocarcinoma MCF-7/0 cells that had been grown in the presence and absence of catechol to gossypol. Exponentially growing MCF-7/0 cells were cultured in the presence of vehicle (▲; MCF-7/0) or 30 μM catechol (●; MCF-7/0/CAT) for 5 days after which time they were harvested and exposed to various concentrations of gossypol for 35 min at 37°C. The cells were then harvested and grown in drug-free growth medium for 15 days. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. Data points are means of triplicate determinations.

Figure 6. Sensitivities of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide in the presence and absence of gossypol. Exponentially growing MCF-7/0 cells were cultured in the presence of vehicle (Δ, ▲; MCF-7/0) or 30 μM catechol (O, ●; MCF-7/0/CAT) for 5 days after which time they were each harvested and incubated with vehicle (Δ, O) or 75 μM gossypol (▲, ●) for 5 min at 37°C. Mafosfamide was added and incubation was continued for an additional 30 min. The cells were then harvested and grown in drug-free growth medium for 15 days. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. Data points are means of triplicate determinations. Cellular levels of aldehyde dehydrogenase activities (cofactor and substrate were 4 mM each of NADP and benzaldehyde, respectively) were 1.8 and 680 $mIU/10^7$ cells in 105,000 g supernatant fractions obtained from Lubrol-treated whole homogenates of MCF-7/0 and MCF-7/0/CAT cells, respectively.

Table 1. Inhibition by gossypol of human aldehyde dehydrogenase-catalyzed oxidation and hydrolysis: IC_{50} values[a]

Enzyme	IC_{50}, μM	
	Oxidation[b]	Hydrolysis[c]
rALDH-1	75	> 200
rALDH-2	45	> 200
nALDH-3	7.5	> 200
tALDH-3	6.6	> 200
GAPDH	70	—
Alkaline phosphatase	—	130

[a]Enzymes were incubated with vehicle or various concentrations of gossypol for 5 min, substrate was added, and initial catalytic rates were quantified as described in Materials and Methods. A minimum of seven gossypol concentrations, at least two of which effected less than 50% inhibition, and two of which effected greater than 50% inhibition, were used. Examples of plots of the primary data thus obtained are shown in Fig. 2. IC50 values were estimated directly from such plots.

[b]Substrate and cofactors were, respectively, acetaldehyde and NAD (4 mM each) for rALDH-1, acetaldehyde (2 mM) and NAD (4 mM) for rALDH-2, benzaldehyde (4 mM) and NAD (1 mM) for the ALDH-3s, and glyceraldehyde 3-phosphate and NAD (1 mM each) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Uninhibited catalytic rates were 0.56, 3.3, 25, 31 and 56 IU/mg protein for rALDH-1, rALDH-2, nALDH-3, tALDH-3 and GAPDH, respectively.

[c]Substrates were *p*-nitrophenyl acetate (500 μM) for the dehydrogenases and *p*-nitrophenyl phosphate (10 mM) for AP. Uninhibited catalytic rates were 126, 476, 11, 9.6 and 16 IU/mg protein for rALDH-1, rALDH-2, nALDH-3, tALDH-3 and alkaline phosphatase, respectively.

Table 2. Inhibition by gossypol of human aldehyde dehydrogenase-catalyzed oxidation: Ki values[a]

Enzyme	Substrate and Cofactor		Km (μM)	Vmax (IU/mg)	Ki (μM)
	Variable (mM)	Fixed (mM)			
rALDH-1	Acetaldehyde (0.125 - 4)	NAD (4)	546	0.66	69
	NAD (0.025 - 1)	Acetaldehyde (4)	59	0.63	5.3
rALDH-2	Acetaldehyde (0.05 - 1)	NAD (4)	5.5[b]	3.4	37
	NAD (0.125 - 4)	Acetaldehyde (2)	327	3.6	7.0
nALDH-3	Benzaldehyde (0.165 - 4)	NAD (1)	413	27	10
	NAD (0.025 - 1)	Benzaldehyde (4)	33	25	0.32
	Benzaldehyde (0.165 - 4)	NADP (4)	389	64	5.8
	NADP (0.5 - 4)	Benzaldehyde (4)	765	72	0.56
tALDH-3	Benzaldehyde (0.125 - 4)	NAD (1)	349	35	4.3
	NAD (0.020 - 1)	Benzaldehyde (4)	47	32	0.10
	Benzaldehyde (0.125 - 4)	NADP (4)	319	69	4.4
	NADP (0.5 - 4)	Benzaldehyde (4)	764	72	0.19

[a]Enzymes were incubated with vehicle or various concentrations of gossypol for 5 min, substrate was added, and initial catalytic rates were quantified as described in Materials and Methods. Representative plots of the primary data from which the kinetic constants were estimated are given in Figs. 3 and 4.

[b]Unlikely to be accurate because it is difficult to ascertain Km values that are less than about 10 μM from the very flat Lineweaver-Burk plots that we generated. Thus, the Km value was determined to be < 0.1 μM when a more appropriate experimental design and method of analysis, viz., integrated Michaelis analysis of a single enzyme-progress curve, was used [Dockham et al., 1992].

Table 3. Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide and phosphoramide mustard in the presence and absence of gossypol[a]

Cell Line	ALDH-3 (mIU/10^7 cells)	Anticancer Agent	Gossypol (μM)	LC_{90} (μM)
MCF-7/0	2	Mafosfamide	0	70
			75	65
MCF-7/0/CAT	680	Mafosfamide	0	> 2000
			75	200
		Phosphoramide mustard	0	1400
			75	1100

[a]Human breast adenocarcinoma MCF-7/0 cells were cultured in the presence of vehicle (MCF-7/0) or 30 μM catechol (MCF-7/0/CAT) for 5 days. At the end of this time, cells were harvested, washed, and resuspended in drug-exposure medium. The cells (1 x 10^5 cells/ml) were then incubated with gossypol or vehicle for 5 min at 37°C after which time various concentrations of mafosfamide, phosphoramide mustard or vehicle were added and incubation was continued as before for 30 min at 37°C. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. LC_{90} values (concentrations of drug required to effect 90% cell-kills) were obtained from plots of log surviving fractions versus concentrations of drug (Fig. 6). Values are means of LC_{90}s obtained in two experiments. Cellular levels of ALDH-3 activity (NADP-linked enzyme-catalyzed oxidation of benzaldehyde; 4 mM each of cofactor and substrate) in 105,000 g supernatant fractions obtained from Lubrol-treated whole homogenates of tumor cells were determined as described in Materials and Methods.

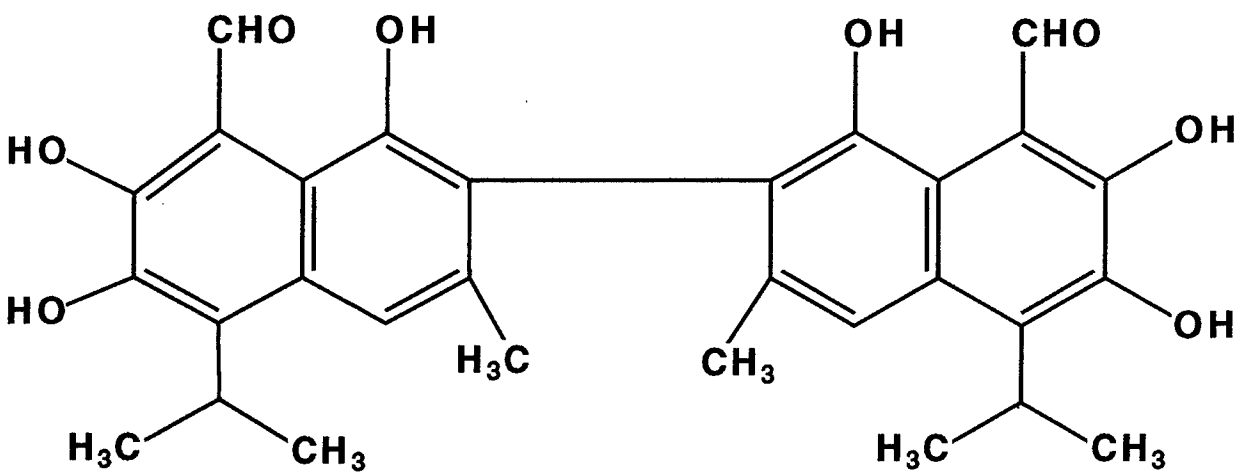


Figure 1.

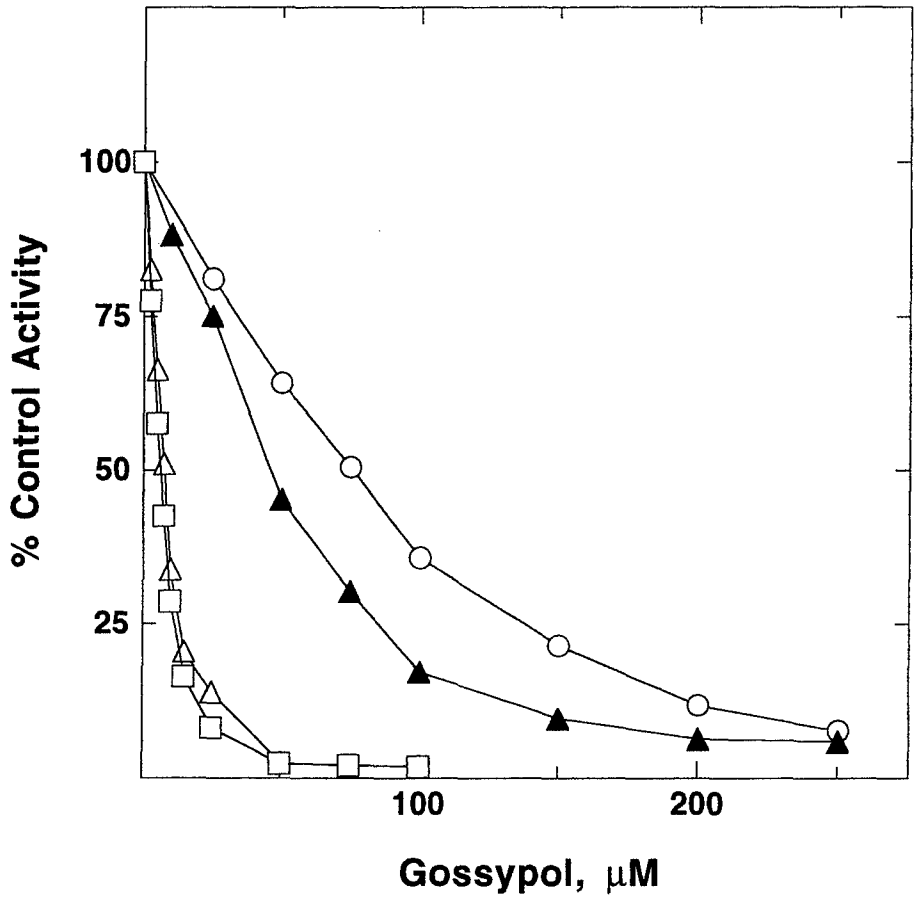
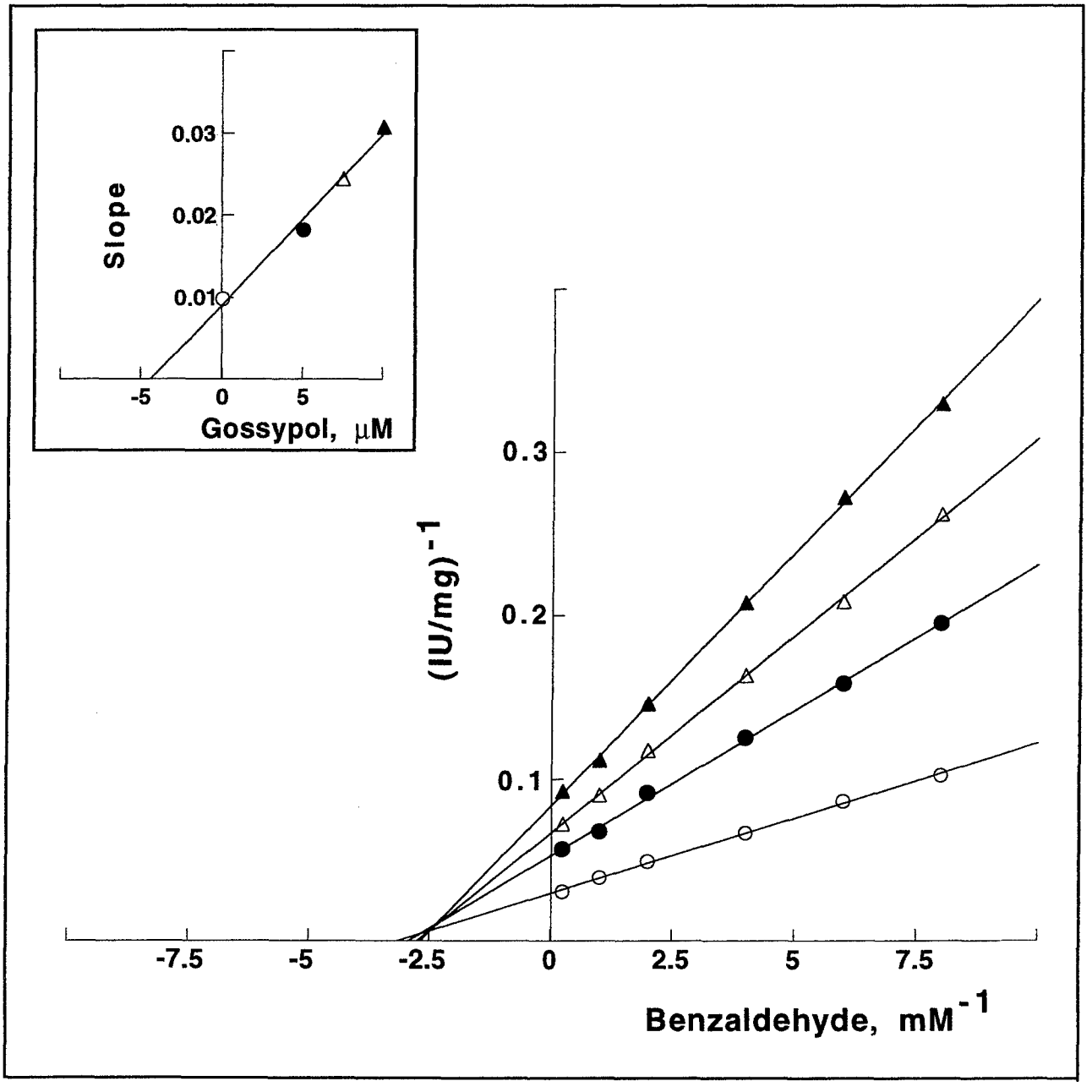


Figure 2.


Slope
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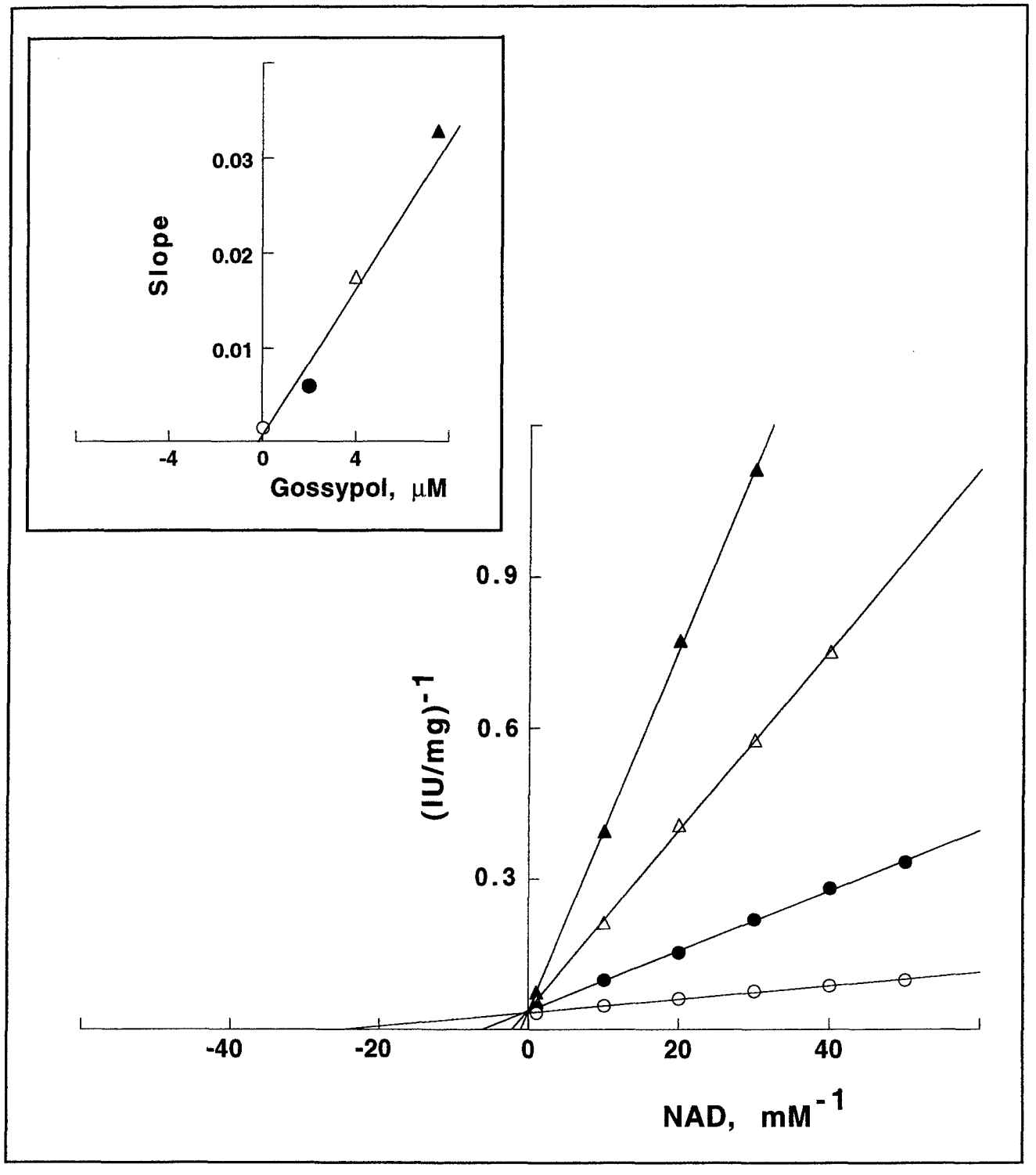
0.02
0.01
-5
0
5
Gossypol, μM
0.3
0.2
0.1
(IU/mg)$^{-1}$
-7.5
-5
-2.5
0
2.5
5
7.5
Benzaldehyde, mM^{-1}

Figure 3.


Slope
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0.01
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0
4
Gossypol, μM
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0.6
0.3
(IU/mg)-1
-40
-20
0
20
40
NAD, mM-1

Figure 4.

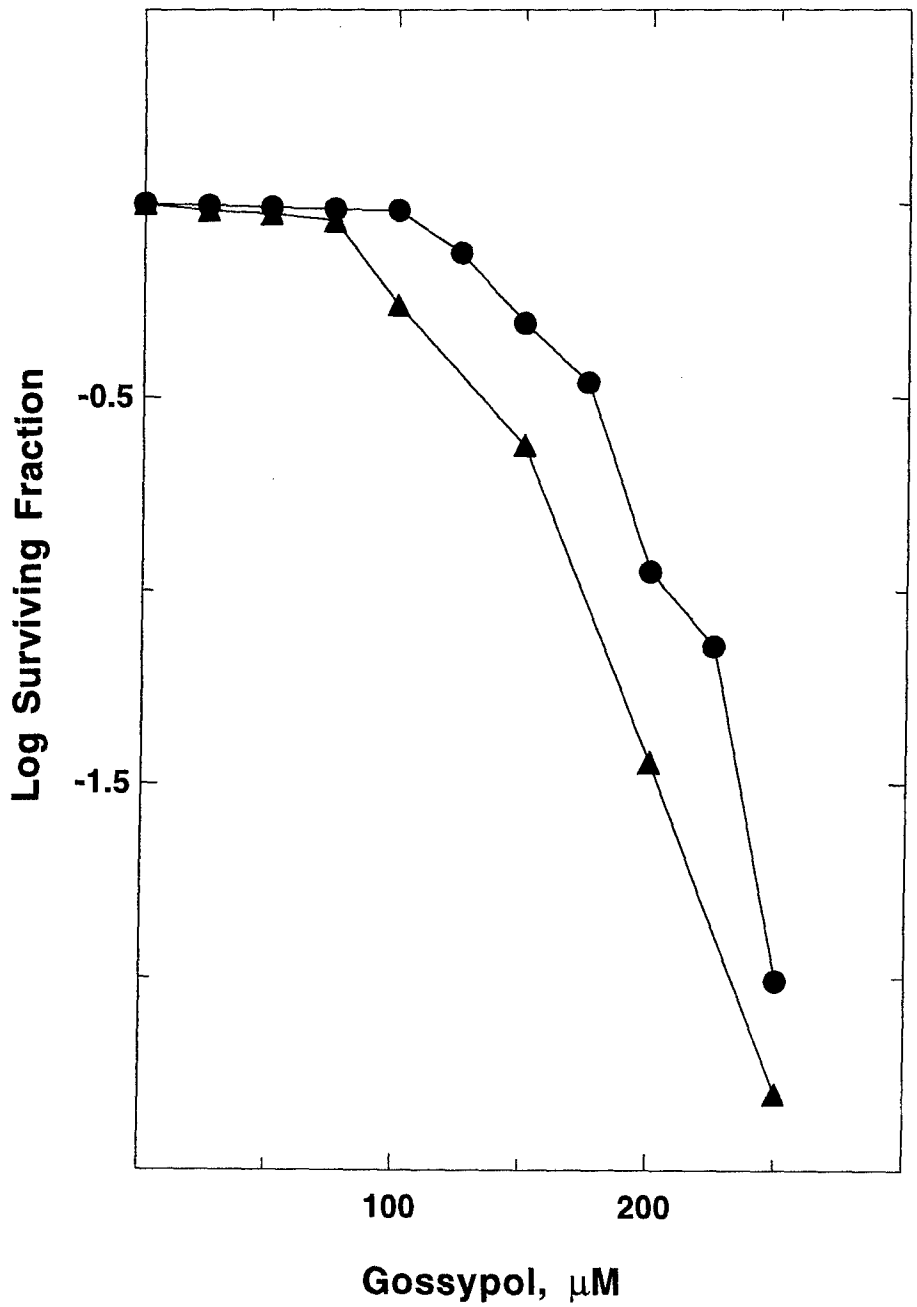

Figure 5.

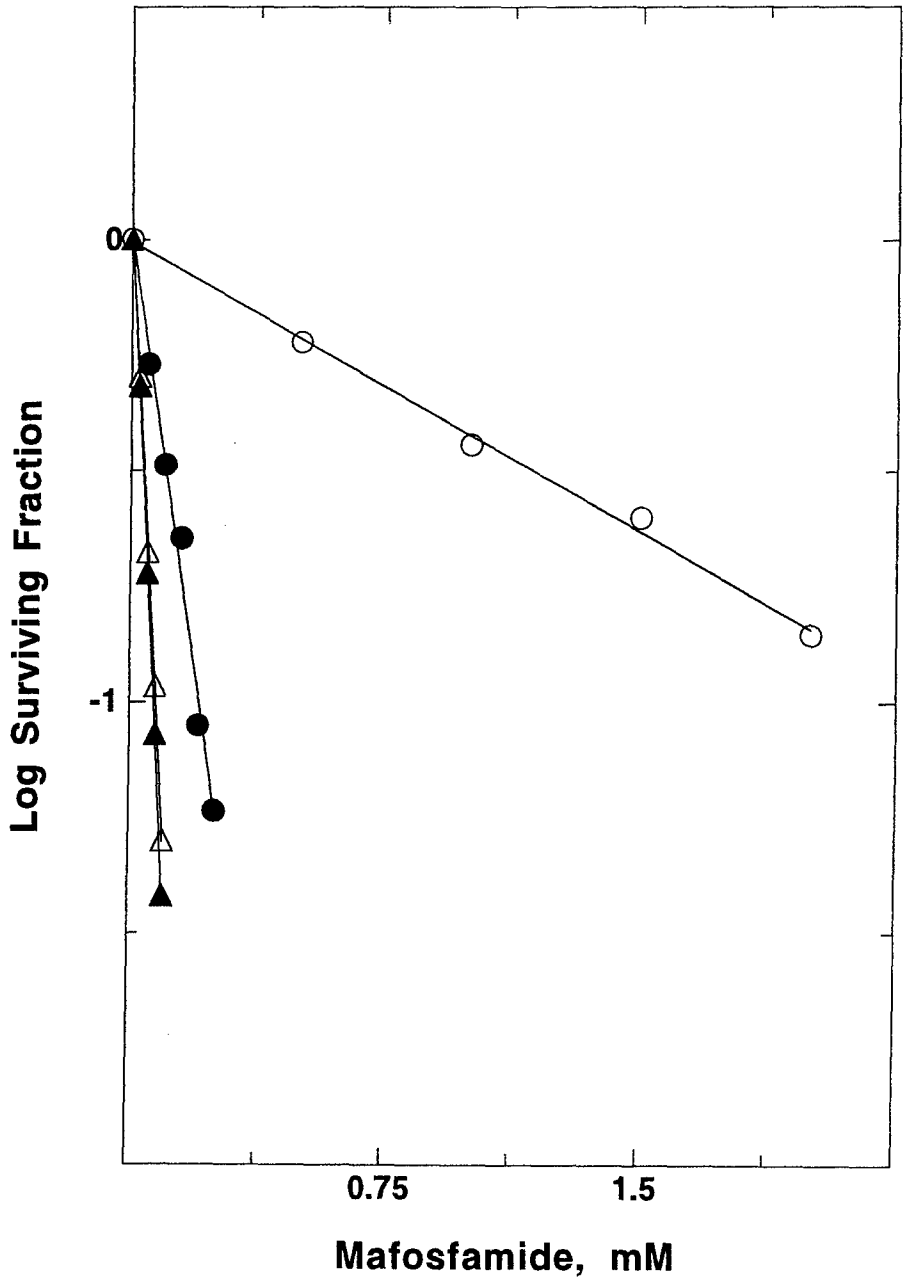

Figure 6.

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Multienzyme-Mediated Stable and Transient Multidrug Resistance and Collateral Sensitivity Induced by Xenobiotics*

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*Supported by USPHS Grant CA 21737 and DOD Grant DAMD 17-94-J-4057.

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FOOTNOTES

[1]Abbreviations: ALDH-3, cytosolic class-3 aldehyde dehydrogenase; GST, glutathione *S*-transferase; DT-D, DT-diaphorase [NAD(P)H:quinone oxidoreductase, NQO1]; UDP-GT, UDP-glucuronosyl transferase; CYP IA1, cytochrome P450 IA1; mIU, milli-International Unit of enzyme activity [nmol NADPH formed/min in the case of aldehyde dehydrogenase activity, nmol of S-(2-chloro-4-nitrophenyl)glutathione formed/min in the case of pan-glutathione *S*-transferase activity, nmol of 2,6-dichlorophenol-indophenol reduced/min in the case of DT-diaphorase activity, nmol of α-naphthyl glucuronide formed/min in the case of UDP-glucuronosyl transferase activity, and nmol of resorufin formed/min in the case of cytochrome P450 IA1 activity]; LC_{90}, drug concentration required to effect a 90% cell-kill; MDR1, multidrug resistance gene 1; MRP, multidrug resistance-associated protein; ABC, ATP-binding cassette; LRP, lung resistance-related protein.

[2]Bifunctional inducers are defined herein as agents that coordinately induce cytosolic class 3 aldehyde dehydrogenase, glutathione *S*-transferases, DT-diaphorase, UDP-glucuronosyl transferase, epoxide hydrolase, dihydrodiol dehydrogenase, carbonyl reductase and cytochrome P450s IA1, IA2 and IB1; xenobiotic responsive elements (XRE) present in the 5'-upstream regions of the genes that code for these enzymes are essential components of the signaling mechanism by which induction is effected [13, 23, 26, 33, 41, 45, 52]. Monofunctional inducers are defined herein as agents that coordinately induce all of the foregoing enzymes except cytochrome P450s IA1, IA2 and (putatively) IB1; in this case, antioxidant responsive elements (ARE) present in the 5'-upstream regions of the genes that code for these enzymes are essential components of the signaling mechanism by which induction is effected [13, 23, 26, 38, 41, 45, 52].

ABSTRACT

Determinants of cellular sensitivity to anticancer drugs include enzymes that catalyze their biotransformation. Coordinated induction of some of these enzymes is known to be effected by a number of dietary constituents, environmental contaminants, pharmacological agents and other xenobiotics, e.g., 3-methylcholanthrene and catechol. Despite the potential for inducing simultaneous changes in tumor cell sensitivity to a wide range of drugs, scant attention has been paid to the impact that dietary constituents and other xenobiotics might have on the therapeutic outcome of cancer chemotherapy. The aim of this investigation was to demonstrate the potential of xenobiotic-induced multienzyme-mediated stable and transient multidrug resistance/collateral sensitivity in a model system. Human breast adenocarcinoma MCF-7/0 cells and a stably oxazaphosphorine-resistant subline thereof, viz., MCF-7/OAP, were grown in the presence of 3-methylcholanthrene (3 μM), catechol (30 μM), or vehicle for 5 days. Relative to their untreated counterparts, 1) MCF-7/0 and MCF-7/OAP cells treated with 3-methylcholanthrene or catechol transiently expressed elevated levels of cytosolic class 3 aldehyde dehydrogenase, glutathione *S*-transferase, DT-diaphorase and UDP-glucuronosyl transferase, and were transiently more a) resistant to mafosfamide, melphalan, and mitoxantrone, and b) sensitive to EO9; and 2) MCF-7/0 and MCF-7/OAP cells treated with 3-methylcholanthrene, but not those treated with catechol, transiently expressed elevated levels of cytochrome P450 IA1 and were transiently more sensitive to ellipticine. Relative to MCF-7/0 cells, MCF-7/OAP cells stably overexpressed all but cytochrome P450 IA1 and were stably more 1) resistant to mafosfamide, melphalan and mitoxantrone, and 2) sensitive to EO9. Inclusion of relatively specific inhibitors of, or alternative substrates for, the enzymes of interest during drug exposure negated the influence of enzyme overexpression on cellular sensitivities to these agents. Untreated, and 3-methylcholanthrene- or catechol-treated, MCF-7/0 and MCF-7/OAP cells were equisensitive to vincristine and nearly so to doxorubicin. Collectively,

these experiments illustrate the potential for both stable and transient xenobiotic-induced multienzyme-mediated multidrug resistance/collateral sensitivity that, although also the result of a single event, is mechanistically different, and that pertains to a largely different group of anticancer agents, than is/does the multidrug resistance effected by cell surface multidrug transporters.

Key words: Aldehyde dehydrogenase, DT-diaphorase, glutathione *S*-transferase, UDP-glucuronosyl transferase, cytochrome P450 IA1, mafosfamide, cyclophosphamide, ifosfamide, oxazaphosphorines, EO9, melphalan, mitoxantrone, ellipticine, doxorubicin, vincristine, multidrug resistance, multidrug collateral sensitivity, breast cancer, monofunctional inducers, bifunctional inducers, phase 1 drug-metabolizing enzymes, phase 2 drug-metabolizing enzymes.

INTRODUCTION

Anticancer drugs, like other pharmacological agents, are often substrates for enzymes that catalyze their conversion to metabolites that are, pharmacologically, either more or less potent than are the compounds from which they originate. Amongst the enzymes that catalyze such conversions are several, viz., ALDH-3[1], GST, DT-D, UDP-GT and CYP IA1, that are known to be coordinately induced by so-called bifunctional inducers[2], e.g., TCDD, indole-3-carbinol and polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and 3,4-benzpyrene, and, except for CYP IA1, by so-called monofunctional inducers, e.g., oltipraz and phenolic antioxidants such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and catechol [reviewed in 3, 6, 7, 26, 45, 52]. Thus, ALDH-3, GST and UDP-GT are known to catalyze the detoxification of oxazaphosphorines, viz., cyclophosphamide, ifosfamide, mafosfamide and 4-hydroperoxycyclophosphamide [reviewed in 45]; melphalan, chlorambucil and phosphoramide mustard [reviewed in 23]; and mitoxantrone [57], respectively, and DT-D and CYP IA1 are known to catalyze the toxification of mitomycin C and the indoloquinone EO9 [reviewed in 39, 58]; and ellipticine [reviewed in 34], respectively.

Although yet to be demonstrated, it follows that when coordinate induction of these enzymes is effected in tumor cells by either monofunctional or bifunctional inducers, cellular sensitivity to relevant antitumor agents should be coordinately altered as well, viz., decreased in those cases where the antitumor drug is converted to a less active metabolite, and increased in those cases where the antitumor drug is converted to a more active metabolite. Cellular sensitivity to such anticancer agents would be expected to return to control levels upon removal of the inducer from the environment, i.e., resistance and collateral sensitivity would each be transient.

Coordinate induction of these enzymes could also be effected by a permanent change (mutation) in a signaling mechanism that upregulates gene transcription and is common to the enzymes of interest. In that case, resistance and collateral sensitivity to relevant antitumor agents would be expected to be stable.

Each of these expectations was fully realized in a human breast adenocarcinoma MCF-7 cultured cell model, thereby illustrating the previously unrecognized potential for both stable and transient, xenobiotic-induced multidrug resistance (and collateral sensitivity) that is mechanistically different, and that pertains to a largely different group of anticancer agents, than is/does the multidrug resistance effected by MRP or the P-glycoprotein coded for by the MDR1 gene.

MATERIALS AND METHODS

Materials

Mafosfamide, melphalan hydrochloride, mitoxantrone hydrochloride and E09 [3-hydroxymethyl-5-aziridinyl-1-methyl-2-(H-indole-4,7-indione)-propenol] were provided by Dr. J. Pohl, Asta Medica AG, Frankfurt, Germany; Dr. G. M. Lyon, Jr., Burroughs Wellcome & Co., Research Triangle Park, NC; Dr. F. E. Durr, American Cyanamid Company, Medical Research Division, Lederle Laboratories, Pearl River, NY; and Dr. H. R. Hendriks, New Drug Development Office, EORTC, Free University Hospital, The Netherlands, respectively. Phosphoramide mustard·cyclohexylamine and doxorubicin hydrochloride were supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and Adria Laboratories Inc., Wilmington, DE, respectively. Purified human GSTs α, μ and π, and affinity-purified polyclonal antibodies specific for each of these isozymes [54], were provided by Dr. A. J. Townsend, Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC. Ellipticine, vincristine sulfate, anti-rabbit IgG alkaline phosphatase conjugate, *p*-nitrophenyl phosphate, α-naphthyl β-D-glucuronide, uridine 5'-diphosphoglucuronic acid (UDP-glucuronic acid), α-naphthol, α-naphthaflavone and CHAPS [(3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate] were purchased from the Sigma Chemical Co., St. Louis, MO. All other chemicals and reagents were obtained from the sources reported in previous publications [46, 48, 51].

Homogenization medium [1.15% (w/v) KCl and 1 mM EDTA in aqueous solution, pH 7.4], drug-exposure medium [horse serum (10%) in a phosphate-buffered saline-based aqueous solution, pH 7.4], growth medium [horse serum (10%) in Dulbecco's Modified Eagle's Medium supplemented with L-glutamine (2 m*M*), sodium bicarbonate (3.7 g/L) and gentamicin (50 mg/L], and Lubrol-treated whole homogenates were prepared as described

previously [46]. Microsomal fractions were also prepared as described previously [51], except that they were ultimately suspended in a 0.25 *M* aqueous sucrose solution when UDP-GT activity was to be quantified.

Cell Culture

Human breast adenocarcinoma MCF-7/0 cells and a stably oxazaphosphorine-resistant subline thereof generated by growing the parent MCF-7/0 cells in the presence of gradually increasing concentrations of 4-hydroperoxycyclophosphamide over a several month period, viz., MCF-7/OAP [18], were obtained originally from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, MA, and were cultured (monolayer) as described previously [46, 48]. Exponentially growing MCF-7/0 and MCF-7/OAP cells were continuously exposed to 3 μ*M* 3-methylcholanthrene or 30 μ*M* catechol for 5 days, as described previously, to obtain MCF-7/0/MC and MCF-7/OAP/MC, and MCF-7/0/CAT and MCF-7/OAP/CAT, cells, respectively [48, 51].

Cultured cells were harvested, resuspended in growth medium, and checked for viability (usually greater than 95% as judged by trypan blue exclusion; preparations exhibiting less than 95% viability were discarded) as described previously [46, 48]. Subsequently, they were again harvested, washed once, and resuspended in drug-exposure medium when cellular sensitivity to the cytotoxic action of various drugs was to be quantified, or in homogenization medium when enzyme activities in microsomal fractions or Lubrol-treated whole homogenates were to be quantified.

Enzyme Assays

ALDH-3, pan-GST, DT-D and CYP IA1 activities, and protein concentrations, were determined as described previously [46, 51]. The spectrofluorometric method described by Mackenzie and Hänninen [1980] was used with modifications to quantify UDP-GT activity. The reaction mixture, minus UDP-glucuronic acid, was prepared at 4°C and

contained 50 m*M* potassium phosphate, 4 m*M* $MgCl_2$, 0.05 m*M* α-naphthol [originally dissolved in 0.25% (v/v) dimethyl sulfoxide in water; the final concentration of dimethyl sulfoxide in the reaction mixture was 0.00625%; this amount of dimethyl sulfoxide did not interfere with the assay], 0.4 m*M* CHAPS and microsomal fractions obtained from 2.5 x 10^6 cells. It was allowed to stand for 10 min at 4°C, after which time it was incubated in a water-bath at 37°C for 5 min. UDP-glucuronic acid was then added to start the reaction (the final UDP-glucuronic acid concentration, reaction volume and pH of the reaction mixture were 2 m*M*, 1 ml and 7.4, respectively). Accumulation of naphthyl glucuronide at 37°C was continuously quantified and recorded with the aid of an automated spectrofluorometer, (Fluorolog-2, SPEX Industries Inc., Edison, NJ) equipped with a circulating-water bath; excitation and emission wavelengths were 293 and 335 nm, respectively. Reagent blanks lacked UDP-glucuronic acid. Authentic α-naphthyl glucuronide was used to prepare standards.

Enzyme-Linked Immunosorbant Assay

GSTs α, μ and π were quantified by an enzyme-linked immuno-sorbant assay (ELISA) [24]. Lubrol-treated homogenates obtained from 1-10 x 10^5 cells were diluted with coating buffer (100 m*M* sodium carbonate in aqueous solution, pH 9.6) and placed into the wells of 96-well microtitration plates (100 μl/well). Adhesion of proteins to the surface area of the wells was effected by incubating the plates in a humidified air-incubator for 2 h at 37°C. The plates were then thoroughly washed with a washing buffer (phosphate-buffered saline-based aqueous solution, pH 7.4, containing 0.05% Tween-20) after which 200 μl of blocking solution (phosphate-buffered saline-based aqueous solution, pH 7.4, containing 0.25% bovine serum albumin and 0.05% Tween-20) were added to each of the coated wells and incubation was continued for 2 h to prevent nonspecific binding of the antibody to the wells. The blocking solution was then removed from the wells, 100 μl aliquots of the primary antibody (antibodies specific for human GSTs α, μ or π) in blocking solution

were added to the blocked wells, and incubation was continued for another 2 h. At the end of this time, the wells were washed with washing buffer, 100 µl aliquots of a secondary antibody (anti rabbit IgG alkaline phosphatase conjugate) in blocking solution were added to the wells, and incubation was continued for yet another 2 h. Following this incubation, the wells were washed thoroughly with washing buffer, 200 µl aliquots of the substrate solution (0.1 m*M* *p*-nitrophenyl phosphate dissolved in Tris-buffered saline-based aqueous solution, pH 9.0) were added to the wells, and incubation at 37°C was effected for 5 to 10 min. Absorbance due to the formation of *p*-nitrophenol (405 nm) was then recorded using a microtitre plate reader (UV-Max Kinetic Microplate Reader, Molecular Devices Corporation, CA) connected to a Macintosh computer equipped with SOFTmax software program. Purified human GSTs α, µ and π (specific activities were 44,500 mIU/mg, 24,100 mIU/mg and 56,700 mIU/mg protien, respectively) were used to generate standard curves.

Drug-Exposure and Colony-Forming Assay

Drug-exposure and the colony-forming assay used to determine surviving fractions were as described previously [46]. Briefly, freshly harvested cells were diluted with drug-exposure medium to a concentration of 1 x 10^5 cells/ml and then exposed to drug or vehicle for 30 (mafosfamide, phosphoramide mustard, melphalan, mitoxantrone, EO9, doxorubicin or vincristine) or 180 (ellipticine) min at pH 7.4 and 37°C after which they were harvested and cultured in drug-free growth medium for 15 (MCF-7/0, MCF-7/0/CAT, MCF-7/OAP, MCF-7/OAP/CAT) or 30 (MCF-7/0/MC and MCF-7/OAP/MC) days. Colonies (≥ 50 cells) were then visualized with methylene blue dye and counted. Stock solutions of EO9 were prepared by first dissolving it in dimethyl sulfoxide and then diluting this solution with drug-exposure medium. Stock solutions of melphalan and ellipticine were prepared by dissolving them in an acidified ethanol solution [5% 0.1 *N* HCl (v/v)]. Stock solutions of mafosfamide, phosphoramide mustard, mitoxantrone,

doxorubicin and vincristine were prepared by placing them into aqueous solution. All drug solutions were prepared just before use. In some experiments, cells were preincubated with benzaldehyde (5 m*M*), dicumarol (25 μ*M*), ethacrynic acid (25 μ*M*), α-naphthol (1 m*M*), α-naphthaflavone (50 μ*M*), or vehicle at 37°C for 5 (all but α-naphthol) or 30 (α-naphthol) min prior to the addition of mafosfamide, EO9, melphalan, mitoxantrone or ellipticine, respectively, after which incubation was continued for 30 or 180 min as before. Stock solutions of benzaldehyde were prepared by dissolving it in water. Stock solutions of dicumarol were prepared by dissolving it in an alkaline (pH 10.0) aqueous solution and then diluting this solution with drug-exposure medium. Stock solutions of ethacrynic acid were prepared by dissolving it in ethanol and then diluting this solution in drug-exposure medium. Stock solutions of α-naphthaflavone and α-naphthol were prepared by dissolving them in dimethyl sulfoxide and then diluting them with drug-exposure medium. The concentration of dimethyl sulfoxide or ethanol ultimately present before and during drug exposure did not exceed 0.1%; this concentration of either solvent did not affect the rate of cell proliferation or induce any of the enzymes of interest. Control experiments established that, at the concentrations used, benzaldehyde, dicumarol, α-naphthol and α-naphthaflavone were without cytotoxic effect against MCF-7/0 and MCF-7/OAP cells. Ethacrynic acid (25 μ*M*) effected a small amount of cell-kill (11%); this was taken into account when calculating the effect of including ethacrynic acid in the drug-exposure medium on LC_{90} values for melphalan.

Data Analysis

Computer-assisted unweighted regression analysis was carried out using the STATView® (Brainpower, Inc., Calabas, CA) statistical program to generate all straight-line functions.

RESULTS

ALDH-3, pan-GST, DT-D, UDP-GT and CYP IA1 levels in untreated, and in 3-methylcholanthrene- and catechol-treated, human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells are given in Table 1. As compared to untreated MCF-7/0 cells, untreated MCF-7/OAP cells and catechol-treated MCF-7/0 cells expressed elevated levels of ALDH-3, pan-GST, DT-D and UDP-GT, but not of CYP IA1, activities, and 3-methylcholanthrene-treated MCF-7/0 cells expressed elevated levels of all five enzyme activities. Similarly, compared to untreated MCF-7/OAP cells, those cultured in the presence of catechol expressed elevated levels of ALDH-3, pan-GST, DT-D, and UDP-GT but not of CYP IA1, activities, whereas those cultured in the presence of 3-methylcholanthrene expressed elevated levels of all five enzyme activities. These findings were entirely as expected given 1) our previous findings, 2) that catechol is a monofunctional inducer, and 3) that 3-methylcholanthrene is a bifunctional inducer [reviewed in 45].

At least three GST isoenzymes, viz., α, μ and π, are present in the cytosol of human cells; each has been shown to be induced in various models by both bifunctional, e.g., 3-methylcholanthrene, and monofunctional, e.g., butylated hydroxyanisole, inducers [reviewed in 23]. Consistent with these reports, the levels of all three isoenzymes were increased when either MCF-7/0 or MCF-7/OAP cells were cultured in the presence of either 3-methylcholanthrene or catechol for 5 days; constitutive levels of all three were, as compared to those in MCF-7/0 cells, higher in MCF-7/OAP cells, Table 1. Interestingly, induction of GST μ in MCF-7/0 cells by 3-methylcholanthrene or catechol was substantially greater than was induction of GSTs α and π. Similarly, expression of GST μ in MCF-7/OAP cells, relative to that in MCF-7/0 cells, was greater than was relative expression of GSTs α and π in these two cell lines.

Sensitivities of untreated, and 3-methylcholanthrene- and catechol-treated, MCF-7/0 and MCF-7/OAP cells to several anticancer drugs are given in Table 2. These findings were essentially also entirely as expected given 1) those presented in Table 1, 2) that ALDH-3, the GSTs and UDP-GT catalyze the detoxification of mafosfamide, melphalan and mitoxantrone, respectively [reviewed in 23, 45; 57], and 3) that DT-D and CYP IA1 catalyze the toxification of EO9 and ellipticine, respectively [reviewed in 34, 58]. Moreover, untreated MCF-7/OAP cells were, relative to untreated MCF-7/0 cells, equisensitive to vincristine and < 2-fold less sensitive to doxorubicin, and MCF-7/0 and MCF-7/OAP cells treated with 3-methylcholanthrene or catechol were, relative to their untreated counterparts, each equisensitive to vincristine and ≤ 2-fold less sensitive to doxorubicin, Table 2. Thus, the relative insensitivity to mafosfamide, melphalan and mitoxantrone exhibited by untreated MCF-7/OAP cells, and 3-methylcholanthrene- and catechol-treated MCF-7/0 and MCF-7/OAP cells, cannot have been effected by the well-known P-glycoprotein-based, or the recently identified MRP-based, multidrug resistance mechanisms since a substantial decrease in cellular sensitivity to vincristine and doxorubicin would be expected if one of these mechanisms was operative. The small decreases in sensitivity to doxorubicin are probably the consequence of increased GST-catalyzed detoxification of this agent [reviewed in 23].

Proportionately, increases in DT-D and CYP IA1 levels were substantially greater than were increases in tumor cell sensitivity to EO9 and ellipticine, respectively. Underlying these discrepancies may be one or more of the following. In addition to the indicated enzymes, other enzymes, the cellular levels of which are also elevated, may be operative; such enzymes would catalyze the formation of metabolites that are inactive or relatively inactive and that cannot give rise to metabolites that are equiactive to those generated by DT-D and CYP IA1. A second possibility is that the cofactor, NAD(P)H in each case, becomes rate-limiting in intact cells. Yet another posssibility is that, while the parent compound is known to be less active than the metabolite generated by the catalytic activity

of the enzymes under investigation, it need not be totally without intrinsic activity and/or unable to give rise to an active metabolite in a reaction catalyzed by another enzyme, the cellular level of which is not caused to be increased by the manipulations used in this investigation. The latter is known to occur in the case of each of these drugs, *vide infra*.

3-Methylcholanthrene- and catechol-induced increases in cellular enzyme levels, as well as changes in sensitivity to the drugs of interest, were transient; all values returned to control values shortly (within two weeks) after the inducing agent was removed from the culture medium [45, 48, 51; and data not presented]. On the other hand, enzyme overexpression was stable in MCF-7/OAP cells, as were the changes in sensitivity to the drugs of interest, i.e., each was retained seemingly indefinitely when the selecting agent was removed from the culture medium [45, 46; and data not presented].

Inhibitors or alternative substrates of/for these enzymes were used to provide additional support for the notion that changes in the catalytic activities indicated did, indeed, account for the altered sensitivites to these drugs (comparing values presented in Table 3 with those presented in Table 2). No attempt was made to use maximally tolerated substrate/inhibitor concentrations or substrate/inhibitor concentrations that would give maximal restoration of sensitivity.

Ostensibly, ALDH-3 catalyzes the detoxification of mafosfamide by catalyzing the irreversible oxidation of its pivotal intermediary aldehyde metabolite, viz., aldophosphamide, to the corresponding acid, viz., carboxyphosphamide [8, 9, 37, 42, 46-49, 51]. Benzaldehyde is a relatively good substrate for ALDH-3 [46, 48]. Thus, acting as an alternative substrate, it should, when included in the drug-exposure medium, prevent the relatively decreased sensitivity to mafosfamide observed in cells expressing relatively increased amounts of ALDH-3 if decreased sensitivity to mafosfamide is the consequence of ALDH-3-catalyzed detoxification of this agent. This is precisely what was observed,

Tables 2 and 3. As expected, given that ALDH-3 levels are very low in untreated MCF-7/0 cells, and that phosphoramide mustard, the metabolite of mafosfamide that effects its cytotoxic action [reviewed in 43] is not a substrate for ALDH-3, inclusion of benzaldehyde in the drug-exposure medium only minimally increased the sensitivity of MCF-7/0 cells to mafosfamide, Tables 2 and 3, and did not alter the sensitivity of any of the cells used in this investigation to phosphoramide mustard [37, 45-48; and unpublished observations]. Benzaldehyde did not inhibit pan-GST-, DT-D-, UDP-GT- or CYP IA1-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to melphalan, EO9, mitoxantrone or ellipticine (data not shown).

GST catalyzes the conjugation of a number of anticancer drugs, e.g., melphalan, to glutathione, thereby detoxifying them [reviewed in 23]. Which of the GST isozymes is/are operative in the case of any given agent is not clear but GST α is almost certainly a major contributor in the case of melphalan [reviewed in 23]. Conjugation of the type under consideration can also occur without the benefit of enzymatic intervention. Ethacrynic acid is known to inhibit GST-catalyzed conjugation reactions, perhaps by serving as an alternative substrate, though irreversible, as well as reversible, binding of this agent to GSTs has been reported [reviewed in 36]. Regardless, given that reduced glutathione levels in the six cell types were virtually identical [45], inclusion of ethacrynic acid in the drug-exposure medium should prevent the relatively decreased sensitivity to melphalan observed in cells expressing relatively increased amounts of GST if decreased sensitivity to melphalan is the consequence of GST-catalyzed detoxification of this agent. This expectation was at least partially realized, Tables 2 and 3. Also as expected, given that GST levels are relatively low in untreated MCF-7/0 cells, inclusion of ethacrynic acid in the drug-exposure medium only marginally increased the sensitivity of MCF-7/0 cells to melphalan. Ethacrynic acid did not inhibit ALDH-3-, DT-D-, UDP-GT- or CYP IA1-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to mafosfamide, EO9, mitoxantrone or ellipticine (data not shown).

DT-D, an obligate two-electron donating enzyme, catalyzes the bioreductive activation of EO9 [reviewed in 58]. Alternatively, bioreductive activation of this agent can occur by the addition of one electron in reactions catalyzed by xanthine oxidase/dehydrogenase, NADPH:cytochrome P450 reductase and cytochrome b_5 reductase. Dicumarol is known to be a potent inhibitor of DT-D-catalyzed reactions [16]. Thus, its inclusion in the drug-exposure medium should prevent the increased sensitivity to EO9 observed in cells expressing relatively increased amounts of DT-D if increased sensitivity to EO9 is the consequence of DT-D catalyzed activation of this agent. This expectation was fully realized, Figure 1 and Tables 2 and 3. Again as expected, given that DT-D levels are relatively low in untreated MCF-7/0 cells, inclusion of dicumarol in the drug-exposure medium only minimally decreased the sensitivity of MCF-7/0 cells to EO9. Dicumarol did not inhibit ALDH-3-, pan-GST-, UDP-GT- or CYP IA1-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to mafosfamide, melphalan, mitoxantrone or ellipticine (data not shown).

A rat liver, 3-methylcholanthrene-inducible, UDP-GT has been shown to catalyze the glucuronidation of mitoxantrone [57], and the human counterpart of this isoenzyme conferred a 10-fold decrease in sensitivity to mitoxantrone when it was transfected into NIH 3T3 cells [10]. These observations are consistent with the notion that the relatively decreased sensitivity to mitoxantrone observed in cells expressing relatively increased amounts of UDP-GT is the consequence of UDP-GT-catalyzed detoxification of this agent. Another possibility, viz., GST-catalyzed detoxification of electrophilic metabolites generated in a CYP IA1-catalyzed reaction [35, 57], could be ruled out because neither ethacrynic acid, nor an inhibitor of CYP IA1-catalyzed reactions, viz., α-naphthaflavone, altered the sensitivities of untreated and treated cells to mitoxantrone (data not shown). Inclusion of α-naphthol, a substrate for UDP-GT [5], in the drug-exposure medium should prevent the relatively decreased sensitivity to mitoxantrone observed in cells expressing relatively increased amounts of this enzyme if decreased sensitivity to mitoxantrone is the

consequence of UDP-GT-catalyzed detoxification of this agent. This expectation was largely realized, Tables 2 and 3. Also as expected, given that UDP-GT levels are very low in untreated MCF-7/0 cells, inclusion of α-naphthol only marginally increased the sensitivity of MCF-7/0 cells to mitoxantrone. α-Naphthol did not inhibit ALDH-3-, pan-GST-, DT-D-, or CYP IA1-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to mafosfamide, melphalan, EO9, or ellipticine (data not shown).

The 9-hydroxy derivative of ellipticine is known to exert much greater antitumor activity than does the parent compound itself. Hydroxylation of ellipticine to 9-hydroxyellipticine is known to be mediated by CYP IA1 and other cytochrome P450s [reviewed in 34]. α-Naphthaflavone is known to inhibit CYP IA1-catalyzed reactions [reviewed in 53]. Thus, its inclusion in the drug-exposure medium should prevent the relatively increased sensitivity to ellipticine observed in cells expressing relatively large amounts of CYP IA1 if increased sensitivity to ellipticine is the consequence of CYP IA1-catalyzed activation of this agent. This expectation was largely realized, Tables 2 and 3. As expected, given that CYP IA1 levels are very low in untreated MCF-7/0 and MCF-7/OAP cells and in catechol-treated MCF-7/0 cells, inclusion of α-naphthaflavone in the drug-exposure medium only marginally decreased the sensitivity of these cells to ellipticine. α-Naphthaflavone did not inhibit ALDH-3-, pan-GST-, or UDP-GT-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to mafosfamide, melphalan, or mitoxantrone (data not shown). However, it did inhibit (90% inhibition at 50 μ*M*) DT-D-catalyzed reduction of 2,6-dichlorophenol-indophenol, and, when included in the drug-exposure medium, largely prevented the increased sensitivity of cells expressing relatively increased amounts of DT-D to EO9. Prevention by α-naphthaflavone of the increased sensitivity to ellipticine exhibited by 3-methylcholanthrene-treated cells cannot be because DT-D catalyzes the bioactivation of ellipticine and α-naphthaflavone inhibits this reaction since 1) dicumarol did not prevent the increased sensitivity of these cells to ellipticine (data

not shown) and 2) sensitivity to ellipticine is not increased in MCF-7/OAP, or catechol-treated MCF-7/0 and MCF-7/OAP, cells, Tables 2 and 3.

These experiments also demonstrate that cellular sensitivity to a given anticancer drug can be restored by the introduction of a suitable substrate/inhibitor.

DISCUSSION

Development of simultaneous resistance to a wide range of drugs is a major impediment to the successful chemotherapy of human tumors.

Multidrug resistance effected by a 170,000 dalton cell surface multidrug transporter (P-glycoprotein; P-170) is a well-known experimental phenomenon and appears to be of clinical relevance [reviewed in 12, 15, 20]. Resistance to the anthracyclines, the *Vinca* alkaloids, the epipodophyllotoxins, actinomycin D and taxol, can be effected by P-170. Relatively elevated cellular levels of P-170 may be intrinsic or the consequence of mutations leading to relevant gene (MDR1) amplification or increased transcription of unamplified MDR1.

Recent studies indicate that multidrug resistance may also be effected by another ABC transporter, namely, MRP [14, 21], and by LRP (p110 major vault protein) [40]. The spectrum of drugs to which resistance may be effected by the 190,000 dalton MRP is similar, but not identical, to that effected by the P-170. Whether multidrug resistance effected by MRP is of clinical significance is yet to be determined but would seem likely given that relatively high levels of it are found in at least some human cancers [11, 17]. The complete spectrum of drugs to which resistance may be effected by LRP is yet to be determined but included appear to be doxorubucin, platinum compounds and at least some nitrogen mustards [25]. Expression of LRP in advanced carcinoma was an indicator of a poor response to platinum- and nitrogen mustard-based chemotherapy [25]. LRP overexpression has also been found to predict a poor response to chemotherapy on the part of acute myeloid leukemia [29].

The findings described herein indicate that simultaneous resistance can also be brought about by the coordinated induction of relevant detoxifying enzymes in target tumor cells,

and that multidrug resistance of this origin is to a group of anticancer drugs largely different than those that are ineffective because of elevated transporter levels.

Like transporter-mediated multidrug resistance, multienzyme-mediated multidrug resistance can be 1) intrinsic (constitutive) and indefinite as in the case of human colon C carcinoma cells [37; and unpublished observations], or 2) slowly acquired and stable as in the case of MCF-7/OAP cells [18, 45, 46].

Unlike transporter-mediated multidrug resistance, multienzyme-mediated multidrug resistance 1) can also be rapidly acquired/induced and transient as in the case of 3-methylcholanthrene- or catechol-treated MCF-7/0 cells, and 2) is accompanied by rapidly acquired/induced and transient multienzyme-mediated multidrug collateral sensitivity, i.e., increased sensitivity to anticancer drugs that are activated by relevant enzymes. Moreover, it can be readily acquired/induced in the absence of exposure to a selecting agent, i.e., to one of the anticancer drugs to which resistance is induced.

As appears to be the case in acquired transporter-mediated multidrug resistance, a single, though different, event, viz., mutation or exposure to an inducer, leads to multienzyme-mediated multidrug resistance/collateral sensitivity.

Multienzyme-mediated multidrug resistance/collateral sensitivity would be to all anticancer drugs that are converted to pharmacologically less/more active metabolites by enzymes, the expression of which is coordinately elevated whether by exposure to bifunctional or monofunctional inducers or as a consequence of a relevant mutation. Indeed, the therapeutic/toxic potential of all drug/chemicals that are converted to biologically less/more active metabolites by these enzymes would be altered upon their elevation. Thus, for example, elevated cellular levels of these enzymes may be of significance in carcinogenesis and chemoprevention since they are known to catalyze the

biotransformation (bioactivation in some cases and bioinactivation in others) of (pro)carcinogens [reviewed in 4, 23, 28, 52, 55, 56].

The molecular alteration(s) underlying the constitutive expression of relatively elevated levels of ALDH-3, GST, DT-D and UDP-GT in MCF-7/OAP cells is unknown. MCF-7/0 cells were cultured in the presence of gradually increasing concentrations of 4-hydroperoxycyclophosphamide for several months to generate the stable oxazaphosphorine-resistant subline termed MCF-7/OAP [18]. Given that 1) 4-hydroperoxycyclophosphamide is a demonstrated mutagen [22], 2) ALDH-3, GST, DT-D and UDP-GT levels are relatively elevated in MCF-7/OAP cells, whereas the level of cytochrome P450 IA1 is not, 3) enzymes coordinately induced by monofunctional inducers include ALDH-3, GST, DT-D and UDP-GT, but not CYP IA1, 4) common to all but CYP IA1 appears to be an antioxidant responsive element (ARE) in the 5'-upstream regions of the genes that code for them [1, 26, 38] and 5) AREs appear to be essential components of the signaling pathway by which monofunctional inducers such as catechol coordinately induce transcriptional activation [reviewed in 26], a mutation giving rise to some permanent change in the signaling pathway in which the ARE participates to regulate gene transcription would seem likely.

Whether multienzyme-mediated multidrug resistance/collateral sensitivity of the type described herein is of clinical significance is not known. However, it is likely that it is since a) simultaneous resistance to several structurally diverse anticancer drugs that effect their cytotoxic action by several different mechansims and that are not removed from cells by multidrug transporters, i.e., P-170 and MRP, is often encountered clinically [19, 25, 32], b) agents known to induce the relevant enzymes in model systems are abundantly present in the diet/environment, e.g., commonly and frequently ingested dietary constituents such as coffee and broccoli as well as brussels sprouts and other members of the *Cruciferae* family of vegetables contain such inducers, c) certain food additives, e.g.,

butylhydroxyanisole, and pharmaceuticals, e.g., oltipraz, are also known to act as inducers of these enzymes in model systems [50 and references cited therein], d) induction of the relevant enzymes in model systems is effected by relatively low doses/concentrations of the inducing agent [48, 50, 51], e) there is evidence indicating that induction of the relevant enzymes by bifunctional and monofunctional inducers occurs in humans, viz., salivary levels of ALDH-3, GSTs and DT-D are coordinately increased in humans that partake of coffee or broccoli [50] and f) relatively large amounts of the relevant enzymes are sometimes found in primary and metastatic human cancer cells, e.g., breast cancer [2, 27, 31, 44]. Interestingly, cellular levels of these enzymes are, on average, apparently constitutively greater in a number of neoplastic tissues when the comparision is with cellular levels in corresponding normal tissues [2, 27, 31] suggesting that permanent upregulation of their expression often occurs during oncogenesis.

Potentially substantial are the clinical ramifications, especially with regard to therapeutic strategies, of multienzyme-mediated multidrug resistance/collateral sensitivity induced by pharmacological and/or dietary/environmental agents. For example, given that maximum induction of the relevant enzymes is achieved rapidly (only a few days after first introducing the inducer) and that enzyme levels return to basal levels rapidly (again, within a matter of a few days after the inducer is removed [45, 48, 50, 51]), certain anticancer drugs may be effective (ineffective) at one point in time, and, with a relevant change in diet, may be ineffective (effective) a few months or even weeks later, i.e., tumor sensitivity (resistance) to such drugs would (appear) to be transient. At least one pharmaceutical, viz., oltipraz, is known to induce the relevant enzymes [reviewed in 3]. It is likely that there are others, hence the potential for numerous, as yet unrecognized, both favorable and unfavorable, drug interactions. In some scenarios, deliberate induction of the relevant enzymes prior to chemotherapy could be of therapeutic benefit; in other scenarios, the desire would be to keep cellular levels of these enzymes at a minimum. Finally, since the thereapeutic effectiveness of certain anticancer drugs would not be independent of certain

dietary constituents and other pharmaceuticals, our findings indicate that both must be taken into consideration when using such agents. In most cases, the choice of an appropriate diet/pharmaceutical agent would be secondary to the choice of an appropriate anticancer drug, but the reverse could also be effected and, in certain scenarios may be the more desirable. Considerations of this type may be especially critical when very high-dose chemotherapy followed by hematopoietic stem cell rescue is to be used, since the desire is to give the maximally tolerated dose and the margin of safety is small. Salivary levels of ALDH-3, GST and DT-D may reflect tissue levels of these and other relevant enzymes [50]. Quantification of the salivary enzymes prior to initiating chemotherapy may be of value with regard to optimization of the chemotherapeutic protocol.

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Table 1. Enzyme activities in methylcholanthrene- and catechol-treated human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells[a]

Cells	Enzyme Activity, mIU/10^7 Cells							
	ALDH-3[b]	GST				DT-D[b]	UDP-GT	CYP IA1[b]
		pan[b]	α	μ	π			
MCF-7/0	2	25	9	13	11	82	0.04	0.03
MCF-7/0/MC	310	150	20	113	34	495	0.78	0.48
MCF-7/0/CAT	768	250	29	121	62	6,395	0.07	0.03
MCF-7/OAP	254	157	27	81	32	340	0.16	0.03
MCF-7/OAP/MC	3,534	192	38	96	36	15,300	1.30	0.67
MCF-7/OAP/CAT	1,593	227	36	135	51	12,400	0.23	0.03

[a]Human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells were cultured in the presence of vehicle (MCF-7/0, MCF-7/OAP), 3 μ*M* methylcholanthrene (MCF-7/0/MC, MCF-7/OAP/MC) or 30 μ*M* catechol (MCF-7/0/CAT, MCF-7/OAP/CAT) for 5 days, and class-3 NADP-dependent aldehyde dehydrogenase (ALDH-3), pan-glutathione *S*-transferase (GST), NADH-dependent DT-diaphorase (DT-D), UDP-glucuronosyl transferase (UDP-GT) and cytochrome P450 IA1 (CYP IA1) activities were quantified as described in Materials and Methods. Substrates were 4 m*M* benzaldehyde, 1 m*M* 1-chloro-2,4-dinitrobenzene, 0.04 m*M* 2,6-dichlorophenol-indophenol, 0.05 m*M* α-naphthol and 0.005 m*M* 7-ethoxyresorufin, respectively. Also quantified at this time were glutathione *S*-transferase α, μ and π levels. ELISAs were used for this purpose as described in Materials and Methods. Values are the means of duplicate or triplicate determinations made in each of at least two separate experiments.

[b]Values are from previous publications [45-48, 51]; they are included here for comparative purposes. Reduced glutathione levels were approximately 185 nmol/10^7 cells for each of the six cell preparations [45].

Table 2. Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells to antineoplastic drugs before and after growing them in the presence of methylcholanthrene or catechol[a]

Cells	LC_{90}, μM						
	MAF	MEL	EO9	MIT	ELP	DXR	VCR
MCF-7/0	60[b]	6	2.1	4	185	2.6	14
MCF-7/0/MC	>2000[b]	15	0.9	75	50	4.0	15
MCF-7/0/CAT	>2000[b]	22	0.5	7	190	5.2	13
MCF-7/OAP	>2000[b]	14	1.2	12	175	4.6	12
MCF-7/OAP/MC	>2000	23	0.1	100	30	5.8	11
MCF-7/OAP/CAT	>2000	20	0.2	17	180	7.0	14

[a]Human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells were cultured in the presence of vehicle (MCF-7/0, MCF-7/OAP), 3 μM 3-methylcholanthrene (MCF-7/0/MC, MCF-7/OAP/MC) or 30 μM catechol (MCF-7/0/CAT, MCF-7/OAP/CAT) for 5 days. At the end of this time, cells were harvested, washed, and resuspended in drug-exposure medium. The cells were then incubated with vehicle or various concentrations of mafosfamide (MAF), melphalan (MEL), EO9, mitoxantrone (MIT), ellipticine (ELP), doxorubicin (DXR) or vincristine (VCR) for 30 (all but ellipticine) or 180 (ellipticine) min at 37°C as described in Materials and Methods. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. LC_{90} values (concentrations of drug required to effect 90% cell-kills) were obtained from plots of log surviving fractions versus concentrations (4-6) of drug. Values are means of two or three separate experiments.

[b]Values are from previous publications [46-48, 51]; they are included here for comparative purposes.

Table 3. Influence of selected enzyme inhibitors/substrates on the sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells to antineoplastic drugs before and after growing them in the presence of methylcholanthrene or catechol[a]

Cells	LC90, μ*M*				
	MAF/BENZ	MEL/EA	EO9/DIC	MIT/NAP	ELP/αNF
MCF-7/0	54[b]	5	2.3	4	200
MCF-7/0/MC	175[b]	9	2.2	15	145
MCF-7/0/CAT	170[b]	11	2.0	3	190
MCF-7/OAP	180[b]	7	2.4	5	175
MCF-7/OAP/MC	220	ND[c]	1.8	20	135
MCF-7/OAP/CAT	200	ND	1.8	6	ND

[a]Human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells were cultured in the presence of vehicle (MCF-7/0; MCF-7/OAP), 3 μ*M* 3-methylcholanthrene (MCF-7/0/MC; MCF-7/OAP/MC) or 30 μ*M* catechol (MCF-7/0/CAT; MCF-7/OAP/CAT) for 5 days. At the end of this time, cells were harvested, washed, and resuspended in drug-exposure medium. The cells were then incubated with benzaldehyde (BENZ, 5 m*M*), ethacrynic acid (EA, 25 μ*M*), dicumarol (DIC, 25 μ*M*), α-naphthol (NAP, 1 m*M*), α-naphthaflavone (αNF, 50 μ*M*) or vehicle for 5 (all but α-naphthol) or 30 (α-naphthol) min at 37°C after which time various concentrations of mafosfamide (MAF), melphalan (MEL), EO9, mitoxantrone (MIT), ellipticine (ELP) or vehicle was added and incubation was continued as before for 30 (all but ellipticine) or 180 (ellipticine) min at 37°C as described in Materials and Methods. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. LC_{90} values (concentrations of drug required to effect 90% cell-kills) were obtained from plots of log surviving fractions versus concentrations (4-6) of drug. Control LC_{90} values (those obtained in the absence of inhibitor) are given in Table 2.

[b]Values are from previous publications [45-48, 51]; they are included here for comparative purposes.

[c]Not determined.

Figure 1. *Influence of dicumarol on the sensitivity of human breast adenocarcinoma MCF-7/0 cells to EO9 before and after growing them in the presence of methylcholanthrene (MCF-7/0/MC) or catechol (MCF-7/0/CAT).* MCF-7/0 cells were grown in the absence (▲, △) or presence of 3 μ*M* 3-methylcholanthrene (◆, ◇) or 30 μ*M* catechol (●, ○) for 5 days, after which time they were harvested and preincubated with vehicle (▲, ◆, ●) or 25 μ*M* dicumarol (△, ◇, ○) for 5 min at 37°C. EO9 was then added and incubation was continued for additional 30 min after which time the cells were harvested and grown in drug-free growth medium for 15-30 days. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. Each point is the mean of measurements on triplicate cultures. DT-diaphorase activities (40 μ*M* dichlorophenol-indophenol; 160 μ*M* NADH) in Lubrol-treated whole homogenates of these cells were 87 (MCF-7/0), 512 (MCF-7/0/MC) and 6,308 (MCF-7/0/CAT) mIU/10^7 cells.

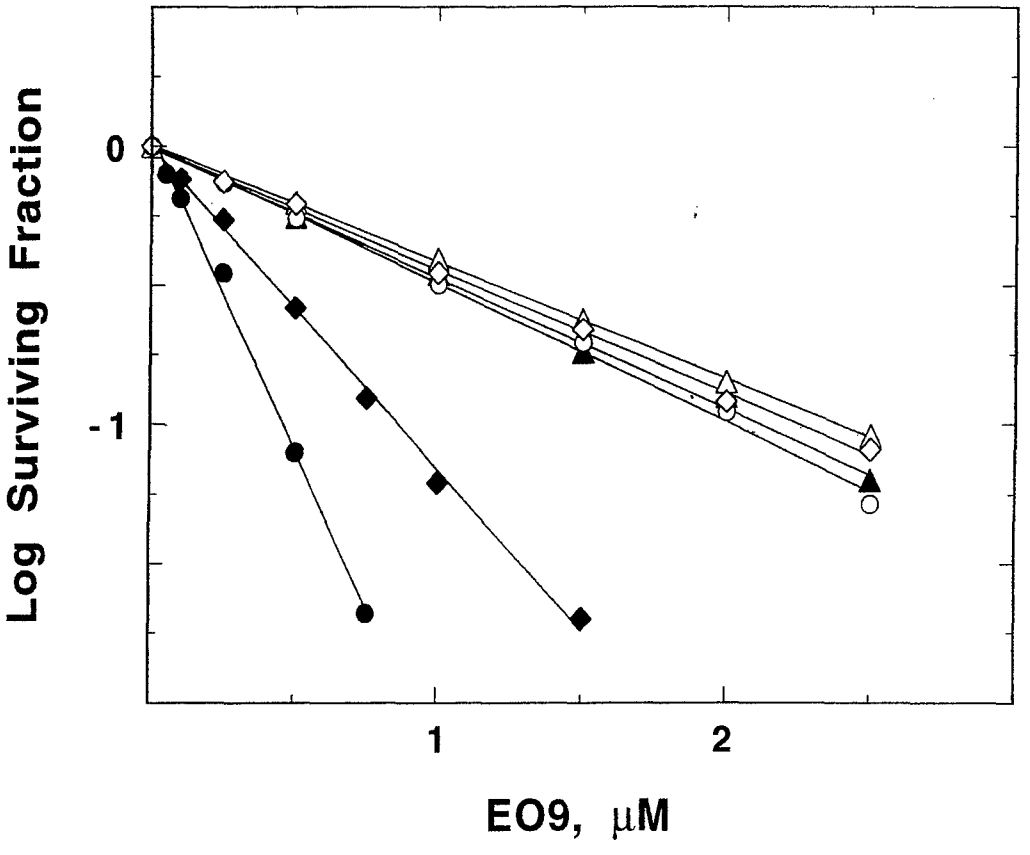

Figure 1.

AOB—MS 426

Pergamon

Archs oral Biol. Vol. 00, No. 0, pp. 000–000, 1995

0003-9969/95 $9.50 + 0.00

S0003-9969(96)00005-2

OVER-EXPRESSION OF GLUTATHIONE S-TRANSFERASES, DT-DIAPHORASE AND AN APPARENTLY TUMOUR-SPECIFIC CYTOSOLIC CLASS-3 ALDEHYDE DEHYDROGENASE BY WARTHIN TUMOURS AND MUCOEPIDERMOID CARCINOMAS OF THE HUMAN PAROTID GLAND

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(Accepted 28 December 1995)

Summary—Cytosolic class-3 aldehyde dehydrogenase (ALDH-3) may help to protect organisms from certain environmental aldehydes by catalysing their detoxification. Consistent with this notion are the reports that relatively high levels of this enzyme are present in tissues, e.g. stomach mucosa and lung, that are so-called ports of entry for such agents. Further, it is found in human saliva. The present investigation revealed that small amounts of this enzyme are also present in human salivary glands; mean values for ALDH-3 activities (NADP-dependent enzyme-catalysed oxidation of benzaldehyde) in cytosolic fractions prepared from submandibular and parotid glands were 52 (range: 29–92) and 44 (range 13–73) miu/g tissue, respectively. Essentially identical or slightly lower levels of this enzyme activity were found in pleomorphic adenomas, an undifferentiated carcinoma, and an adenocystic carcinoma, of the parotid gland. On the other hand, Warthin tumours, and mucoepidermoid carcinomas of the parotid gland exhibited relatively elevated levels of ALDH-3 activity; mean values were 1200 (range: 780–1880) and 810 (range: 580–1200) miu/g tissue, respectively. The ALDH-3 found in normal salivary glands was, as judged by physical, immunological and kinetic criteria, identical to human stomach mucosa ALDH-3 whereas the ALDH-3 present in Warthin tumours and mucoepidermoid carcinomas of the parotid appeared to be a subtle variant thereof. Qualitatively paralleling the relatively elevated ALDH-3 levels in mucoepidermoid carcinomas and Warthin tumours were relatively elevated levels of glutathione S-transferase (α and π) and DT-diaphorase levels. As was the case with ALDH-3, glutathione S-transferase (α and π) and DT-diaphorase levels were not elevated in pleomorphic adenomas. Glutathione S-transferase μ was not detected in the two normal parotid gland samples, or in the single pleomorphic adenoma sample tested. It was found in the single mucoepidermoid carcinoma sample, and in one of the two Warthin tumour samples. Cellular levels of ALDH-3, glutathione S-transferases and/or DT-diaphorase could be a useful criteria when the decision to be made is whether a salivary gland tumour is a mucoepidermoid carcinoma. ALDH-3 and glutathione S-transferases are known to catalyse the detoxification of two agents that are used to treat salivary gland tumours, *viz.* cyclophosphamide and displatin, respectively. Thus elevated levels of these enzymes in the mucoepidermoid carcinomas must account for, or at least contribute to, the relative ineffectiveness of these agents when used to treat this tumour.

Key words: aldehyde dehydrogenase, glutathione S-transferase, DT-diaphorase, salivary glands, parotid glands, salivary gland tumours, Warthin tumours, mucoepidermoid carcinomas, cyclophosphamide, aldophosphamide, oxazaphosphorines.

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Abbreviations: ALDH-3, cytosolic class-3 aldehyde dehydrogenase; miu, milli-international unit of enzyme activity (nmol NAD(P)H formed/min in the case of aldehyde dehydrogenase activity, nmol of the conjugate of 1-chloro-2,4-dinitrobenzene and glutathione formed/min in the case of glutathione S-transferase activity, nmol of 2,6-dichlorophenol-indophenol reduced/min in the case of DT-diaphorase activity, and nmol *p*-nitrophenol formed/min in the case of esterase activity). PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

INTRODUCTION

Cytosolic class-3 aldehyde dehydrogenase (ALDH-3) is currently the subject of extensive investigation by our laboratory because it is a demonstrated determinant of cellular sensitivity to certain widely used anticancer drugs, *viz.* cyclophosphamide, ifosfamide, 4-hydroperoxycyclophosphamide and mafosfamide (cellular sensitivity to these agents decreases as the cellular concentration of ALDH-3 increases) (Sladek, 1993, 1994; Sreerama and Sladek, 1993a, b, 1994a; Bunting, Lindahl and Townsend, 1994; Rekha, Sreerama and Sladek, 1994; Sreerama, Rekha and Sladek, 1995a). ALDH-3, like other aldehyde dehy

drogenases, is a bifunctional enzyme in that it catalyses the oxidation of aldehydes as well as the hydrolysis of esters (Sreerama and Sladek, 1994b; Sladek, Sreerama and Rekha, 1995). Hepatic levels of this enzyme are ordinarily very low or even nil, but substantial amounts of it are found in certain other cells, especially those that line the alimentary canal; thus, high levels of this enzyme are found in stomach mucosa, and lower, but still substantial, levels are found in the small- and large-intestinal mucosa (Sreerama and Sladek, 1993a, b, and references cited therein). It is also found in human saliva (Sreerama, Hedge and Sladek, 1995). Additionally, it is present at high levels in several tumour cell lines that exhibit intrinsic or acquired resistance to mafosfamide and other oxazaphosphorines (Sreerama and Sladek, 1993b, 1994a; Fekha *et al.*, 1994; Sreerama *et al.*, 1995a). Interestingly, the enzyme found in tumour cells appears to be a slight variant of the one found in normal cells (Sreerama and Sladek, 1994b; Sreerama and Sladek, 1995).

Endogenous substrates for ALDH-3 have yet to be identified and its biological role is uncertain if not unknown. Its function may be to detoxify xenobiotics. Additionally or alternatively, its function may be to catalyse the detoxification of otherwise toxic aldehydes arising from lipid peroxidation, thereby protecting cells in which the enzyme is present and such aldehydes arise (reviewed in Lindahl, 1992).

Cellular levels of ALDH-3 and certain other enzymes, *viz.*, glutathione S-transferases and DT-diaphorase, can be coordinately induced by various environmental contaminants, e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin, TCDD) and polycyclic aromatic hydrocarbons such as methylcholanthrene (Lindahl, 1992; Sreerama and Sladek, 1993b, 1994a), as well as by many dietary constituents, e.g., phenolic antioxidants such as catechol and *tert*-butylhydroquinone (Sreerama *et al.*, 1995a).

Recently, we reported the presence of ALDH-3, glutathione S-transferases μ, μ and π, and DT-diaphorase in human saliva, and that salivary levels of these enzymes were elevated in individuals consuming relatively large quantities of coffee and broccoli (Sreerama *et al.*, 1995b). The origin of these enzymes in saliva was not unequivocally established, but a possibility is the salivary glands because glutathione S-transferases α, μ and π are known to be present in normal salivary glands (Corrigall and Kirsch, 1988; Campbell *et al.*, 1991; Zeiper *et al.*, 1994). Glutathione S-transferase π is also reportedly present in salivary gland tumours (Campbell *et al.*, 1991; Zeiper *et al.*, 1994). Further supporting this notion, preliminary investgigations in our laboratory revealed the presence of ALDH-3 activity in normal salivary glands as well as in salivary gland tumours. Of interest, also, was that cellular levels of this activity were sometimes highly elevated in certain salivary gland tumours. The results of these and additional investigations now directed us towards the quantification of ALDH-3, as well as glutathione S-transferase and DT-diaphorase, activities in normal and neoplastic salivary gland tissue. Also reported is additional evidence supporting the notion that the ALDH-3 present in tumour cells is a slight variant of the one found in normal cells.

MATERIALS AND METHODS

4-Hydroperoxycyclophosphamide was supplied by Dr J. Pohl, Asta-Medica AG, Frankfurt, Germany. Purified human glutathione S-transferases α, μ and π and affinity-purified polyclonal antibodies specific for each of these isozymes (Townsend *et al.*, 1989) were provided by Dr A. J. Townsend, Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC. All other chemicals, reagents and supplies were purchased from commercial sources or prepared as described previously (Sreerama and Sladek, 1993a, 1994a; Sreerama *et al.*, 1995b).

Human normal stomach mucosa, human normal salivary glands (submandibular and parotid) and human salivary gland tumours were obtained from the Cooperative Human Tissue Network, Midwestern Division, Columbus, OH.

Purification of ALDH-3s from various sources and preparation of chicken anti-stomach ALDH-3 IgY were according to protocols described previously (Sreerama and Sladek, 1993a; Sreerama *et al.*, 1995b).

Soluble (105,000 *g* supernatant) fractions of normal salivary glands and salivary gland tumours were prepared as described previously for lung and placenta (Sreerama and Sladek, 1993a) except that the homogenization medium was 1.15% (w/v) KCl and 1 mM EDTA in aqueous solution, pH 7.4, and tumours containing excessive connective tissue were minced well in homogenization medium before homogenization in a Dounce homogenizer with the aid of an Omni-mixer (Sorvall, model 17105, Norwalk, CT). Soluble (105,000 *g* supernatant) fractions thus obtained were used as such when aldehyde dehydrogenase, glutathione S-transferase and DT-diaphorase activities present therein were to be quantified. They were further processed when they were to be submitted to SDS–PAGE or isoelectric focusing or when column chromatographic purification of ALDH-3 was to be attempted, *viz.* they were desalted with the aid of PD-10 (Sephadex G-25) columns (isoelectric focusing, column chromatographic purification) and/or concentrated with the aid of Centricon-10 (Amicon Division, W. R. Grace & Co., Danvers, MA) concentrators by low-speed centrifugation (isoelectric focusing, column chromatographic purification, SDS–PAGE). Microsomal fractions were prepared as described previously (Sreerama *et al.*, 1995a), except that final suspension was in 250 mM sucrose solution when uridine diphosphate glucuronosyl transferase activity was quantified.

Spectrophotometric assays for aldehyde dehydrogenase, esterase, glutathione S-transferase and DT-diaphorase activities; determination of protein concentrations; non-denaturing-gradient PAGE; SDS–PAGE; isoelectric focusing; protein visualization; and staining for aldehyde dehydrogenase activity were as described by Sreerama and Saldek (1993a, 1994a). Preliminary experiments with purified enzymes established that ALDH-3s (whether of normal or tumour cell origin) but not ALDH-1, catalysed NADP-dependent oxidation of benzaldehyde. Uridine diphosphate-glucuronosyl transferase and cytochrome P450 IA1/2 activities were quantified spectrofluorimetrically as described previously (Mackenzie and Hanninen, 1980; Sreerama *et al.*, 1995a). Electrotransfer and immunoblot analysis of ALDH-3s and glutathione S-transferases were also essentially as described before (Sreerama and Sladek, 1993a; Townsend *et al.*, 1989); antibody dilutions were 1:500 and 1:1000 in the case of ALDH-3 and glutathione S-transferases, respectively.

Double-reciprocal plots of initial rates compared to substrate concentrations were used to estimate all K_m values. Initial rates were determined in duplicate for each of the six to eight substrate concentrations used to generate each value. Wilkinson weighted linear regression analysis (Wilkinson, 1961) was used to fit the lines to the double-reciprocal plot values.

Computer-assisted unweighted regression analysis was carried out using the STATView (Brainpower, Inc., Calabas, CA) statistical program to generate all other linear functions.

RESULTS

Initial studies revealed that soluble (105,000 *g* supernatant) fractions prepared from normal submandibular and parotid glands catalysed NADP-dependent oxidation of benzaldehyde to benzoic acid (Table 1), as well as NAD-dependent oxidation of acetaldehyde to acetic acid (data not presented), albeit at low rates. NADP-dependent enzyme-catalysed oxidation of benzaldehyde to benzoic acid was always much higher in certain parotid gland neoplasms, *viz.* Warthin tumours and mucoepidermoid carcinomas (Table 1). It was not higher in certain other neoplasms, viz. pleomorphic adenomas, an undifferentiated carcinoma and an adenocystic carcinoma. Enzyme activity was essentially confined to the 105,000 *g* supernatant fractions, i.e., only negligible amounts of these activities were present in Lubrol-solubilized particulate (105,000 *g* pellet) fractions (data not presented).

Isoelectric focusing of the proteins present in soluble (105,000 *g* supernatant) fractions of the above samples followed by staining for aldehyde dehydrogenase activity (cofactor was NAD; substrates were benzaldehyde, octanal and acetaldehyde) revealed banding patterns characteristic of two aldehyde dehydrogenases, *viz.* ALDH-3 (two to four bands within the pI range 5.7–6.4) and ALDH-1 (pI 5.2) (data not shown); in all cases ALDH-3 was, relative to ALDH-1, present in great excess (>15-fold). In the case of Warthin tumours, two to three bands were present within the pI range 5.7–6.4. In the case of normal tissues as well as all other neoplastic tissues, four bands were seen within this pI range. Similar experiments with Lubrol-solubilized 105,000 *g* pellet fractions obtained from the above-mentioned normal and neoplastic salivary gland tissues revealed the presence of a single aldehyde dehydrogenase, *viz.* ALDH-2 (pI 4.9) (data not shown).

Also present in 105,000 *g* supernatant fractions obtained from normal salivary glands were easily measurable levels of glutathione S-transferase and DT-diaphorase activities (Table 2). Qualitatively paralleling the elevated level of ALDH-3 activity in Warthin tumours and mucoepidermoid carcinomas were elevated levels of glutathione S-transferase and DT-diaphorase activities. Glutathione S-transferase and DT-diaphorase activities, like ALDH-3 activity, were not elevated in a pleomorphic adenoma of the parotid gland.

Constitutively elevated levels of ALDH-3, glutathione S-transferase, DT-diaphorase, as well as of uridine diphosphate-glucuronosyl transferase and cytochrome P450 IA1/2, activities in Warthin tumours or mucoepidermoid carcinomas would be

Table 1. Enzyme activity (NADP-dependent catalysis of benzaldehyde oxidation) in 105,000 *g* supernatant fractions prepared from normal and neoplastic salivary glands[a]

Salivary gland	Microscopic diagnosis	*n*	Enzyme activity (miu/g tissue) Range	Mean ± SD
Submandibular	Normal	6	29–92	52 ± 24
Parotid	Normal	7	13–73	44 ± 22
	Pleomorphic adenoma[b]	6	3–70	45 ± 22
	Warthin tumour[b]	13	730–1880	1200 ± 370
	Undifferentiated carcinoma	1	–	12
	Adenocystic carcinoma	1	–	24
	Mucoepidermoid carcinoma	6	580–1200	810–220

[a]Benzaldehyde (4 mM) and NADP (4 mM) were used as the substrate and cofactor, respectively, to quantify enzyme activity.

[b]Pleomorphic adenomas with Warthin tumours are benign; all other tumours are malignant.

Table 2. ALDH-3, glutathione S-transferase and DT-diaphorase activities in 105,000 *g* supernatant fractions prepared from normal and neoplastic salivary gland tissues

		Enzyme activity (miu/g tissue)		
Salivary gland	Microscopic diagnosis	ALDH-3	Glutathione S-transferase	DT-diaphorase
Submandibular	Normal	45	2680	580
Parotid	Normal	68	3100	520
	Normal	54	3200	740
	Pleomorphic adenoma	50	2200	790
	Warthin tumour	1450	14,200	2300
	Warthin tumour	1680	16,600	4100
	Mucoepidermoid carcinoma	1200	10,500	1500

Each value is the mean of three determinations on each tissue sample.

consistent with the notion of stable up-regulation of a signalling/regulatory pathway governing the expression of all these enzymes, *viz.* regulation via Ah receptors/xenobiotic responsive elements (Sladek *et al.*, 1995, and references cited therein). On the other hand, constitutively elevated levels of all but cytochrome P450 IA1/2 and, perhaps, uridine diphosphate-glucuronosyl transferase, activities would be consistent with the notion of stable up-regulation of a signalling/regulatory pathway, *viz.* regulation via antioxidant responsive elements, governing the expression of ALDH-3, glutathione S-transferases, DT-diaphorase and, perhaps, uridine diphosphate-glucuronosyl transferase, but not cytochrome P450s IA1/2 (Sladek *et al.*, 1995, and references cited therein). We did not detect any uridine diphosphate-glucuronosyl transferase or cytochrome P450 IA1/2 activities in microsomal fractions prepared from homogenates of either normal parotid glands or Warthin tumours and mucoepidermoid carcinomas of parotid glands (data not shown). However, the tumour samples had been stored at −70°C for more than 8 months and microsomal enzymes are known to be unstable on prolonged storage at this temperature (reviewed in Okey, 1990).

As judged by immunoblot analysis, glutathione S-transferases α and π were present in each of the two normal parotid glands examined and the level of each was elevated in the mucoepidermoid carcinoma and the two Warthin tumours, but not in the pleomorphic adenoma (Fig. 1). In contrast, glutathione S-transferase μ was not detected in either of the two normal glands, or in the pleomorphic adenoma. It was found in the mucoepidermoid carcinoma and in one of the two Warthin tumours. Shown in this figure, too, is that, as judged by immunoblot analysis also, ALDH-3 is present at relatively elevated levels in the Warthin tumours and mucoepidermoid carcinoma, whereas the ALDH-3 level in a pleomorphic adenoma is not

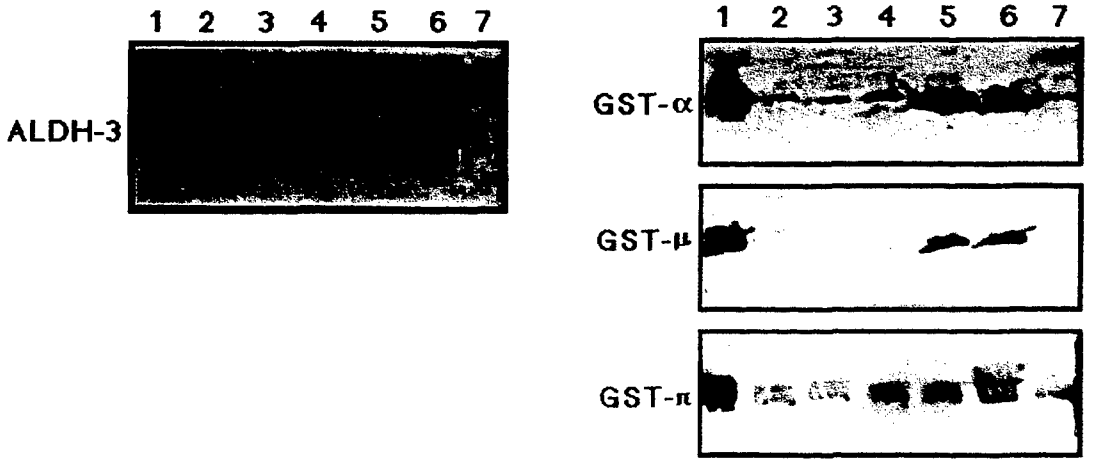

Fig. 1. Immunoblot analysis of 105,000 *g* supernatant fractions prepared from normal parotid glands and several parotid gland tumours for the presence of ALDH-3 and glutathione S-transferases (GST) α, μ and π. Tissue samples evaluated are those for which global enzyme activity is given in Table 2. One microgram each of authentic stomach mucosa ALDH-3 (left panel, lane 1) and glutathione S-transferases α, μ and π (right panels, lane 1), and 105,000 *g* supernatant fractions (100 μg each) prepared from normal parotid glands (lanes 2 and 3), Warthin tumours of parotid glands (lanes 4 and 5), a mucoepidermoid carcinoma of the parotid gland (lane 6) and a pleomorphic adenoma of the parotid gland (lane 7) were first subjected to SDS–PAGE. Proteins thus resolved were then electrotransferred to Immobilon-PVDF transfer membranes and probed with antibodies against human ALDH-3 and glutathione S-transferases α, μ and π. Anti-human stomach mucosa ALDH-3 IgY and anti-human liver glutathione S-transferases α, μ and π IgG were prepared and used as described.

Table 3. ALDH-3s purified from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands

Source	Specific activity (miu/mg protein)	Yield (%)	Fold-purification
Normal parotid gland	31,800	80	30,600
Warthin tumours	32,000	55	2920
Mucoepidermoid carcinomas	32,800	67	5330

ALDH-3s were purified. Benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively, to monitor aldehyde dehydrogenase activity. The specific activities of the purified human stomach musoca and salivary ALDH-3s used elsewhere in these investigations were 33,000 and 31,000 miu/mg under identical assay conditions (Sreerama and Sladek, 1993a; Sreerama *et al.*, 1995b).

elevated, when the comparison is with levels of this enzyme in normal parotid glands.

Evidence to support or refute the notion that the ALDH-3 present in tumour tissues is a variant of that present in normal tissues was sought next. Purified enzymes were needed for this purpose. The purification procedures have been used by us previously to successfully separate ALDH-3 from other aldehyde dehydrogenases and to obtain apparently pur ALDH-3 from various sources (Sreerama and Sladek, 1993a, 1994a, b; Rekha *et al.*, 1994; Sreerama *et al.*, 1995a, b). Specific activities of the apparently pure aldehyde dehydrogenases isolated from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands, are given in Table 3.

The physical (a native molecular weight of 110,000 as judged by non-denaturing linear gradient PAGE, data not presented; a subunit molecular weight of 54,500 (Fig. 2); recognition of the denatured enzyme by anti-stomach mucosa ALDH-3 IgY, Fig. 3) and catalytic (substrate and cofactor preferences as judged by K_m values, Tables 4 and 5; esterolytic activity, Table 6) properties of the ALDH-3s purified from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands, were essentially identical to those reported previously for stomach mucosa and salivary ALDH-3s and for ALDH-3s purified from human breast adenocarcinoma MCF-7, and human colon C carcinoma, cells (Sreerama and Sladek, 1993a, 1994a; Rekha *et al.*, 1994; Sreerama *et al.*, 1995a, b).

Although the banding patterns obtained on subjecting ALDH-3s purified from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands, to isoelectric focusing were not exactly identical to the banding patterns obtained with stomach mucosa or salivary ALDH-3s (Fig. 4), or with human breast adenocarcinoma MCF-7, or human colon C carcinoma, cells

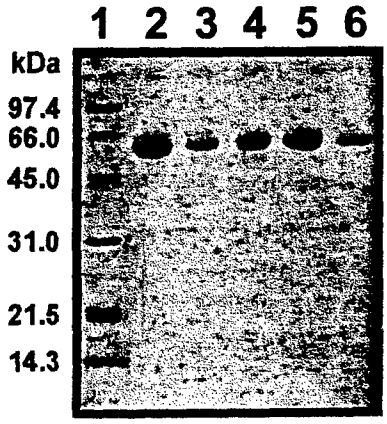

Fig. 2. Subunit molecular weight of ALDH-3s purified from normal parotid glands and Warthin tumours and mucoepidermoid carcinomas of parotid glands, as determined by SDS–PAGE. Purification and SDS–PAGE of ALDH-3s were as described. Subjected to SDS–PAGE were molecular-weight markers (lane 1), and 3–5 μg of each of the ALDH-3s purified from stomach mucosa (lane 2), normal parotid glands (lane 3), Warthin tumours of parotid glands (lane 4), mucoepidermoid carcinomas of parotid glands (lane 5) and saliva of a healthy adult human (lane 6). Molecular-weight markers were lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin monomer (66 kDa) and phosphorylase *b* (97.4 kDa). Proteins in each lane were visualized by staining with Coomassie Brilliant Blue R-250. A plot of log M_r against mobility was used to estimate subunit molecular weights.

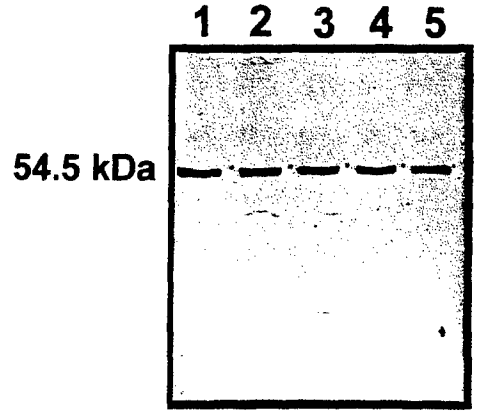

Fig. 3. Immunoblot visualization of ALDH-3s purified from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands, and subjected to SDS–PAGE. Purification and SDS–PAGE of ALDH-3s (1 μg each) were exactly as described in the legend to Fig. 2. Electrotransfer of proteins to an Immobilon PVDF-transfer membrane was as described in text. Anti-stomach mucosa ALDH-3 IgY was generated and used to visualize ALDH-3s purified from stomach mucosa (lane 1), normal parotid glands (lane 2), Warthin tumours of parotid glands (lane 3), mucoepidermoid carcinomas of parotid glands (lane 4) and saliva of a healthy adult human (lane 5).

Table 4. Substrate preferences of ALDH-3s purified from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands

		K_m (μM)				
Substrate (mM)	Cofactor[a]	Stomach[b] mucosa	Whole[b] saliva	Parotid glands	Warthin tumours	Mucoepidermoid carcinomas
Benzaldehyde (0.05–4)	505	465	450	469		525
	NADP	486	463	460	462	510
Acetaldehyde (25–200)	NAD	80,000	85,000	82,000	78,000	79,000
	NADP	81,000	85,000	83,000	87,000	81,000

[a]NAD and NADP concentrations were 1 and 4 mM, respectively.
[b]K_m values for stomach mucosa and salivary ALDH-3s are from previous publications (Sreerama and Sladek, 1993a; Sreerama *et al.*, 1995b); they are included here for comparative purposes.

(Sreerama and Sladek, 1993a, 1994a; Rekha *et al.*, 1994; Sreerama *et al.*, 1995b), in that the number and/or relative staining intensity of the bands differed somewhat, they were all characteristic of class-3 aldehyde dehydrogenases in that the pI values were all in the range 5.7–6.4; it is well known that isoelectric focusing patterns of class-3 aldehyde dehydrogenases, whether of normal, or tumour, cell origin, vary somewhat with the tissue of origin in that as few as two, and as many as five, bands can be present, and/or that the relative amounts of each band can vary, but the pI values always fall in the range 5.7–6.4 (reviewed in Goedde and Agarwal, 1990; Sreerama and Sladek, 1993a).

Like all other ALDH-3s (Sreerama and Sladek, 1993a, 1994a; Rekha *et al.*, 1994; Sreerama *et al.*, 1995a, b), each of the ALDH-3s under current investigation were only partially (<30%) inhibited by a high concentration of disulfiram (50 μM) and were heat labile, i.e., the catalytic activities of each of the preparations were completely lost within 10 min of incubation at 56°C (data not presented).

As compared to ALDH-3s isolated from tumour cells (human breast adenocarcinoma MCF-7 and human colon C carcinoma cells), ALDH-3s isolated from normal cells/fluids, (human stomach mucosa and human saliva) catalyse the oxidation of aldophosphamide to carboxyphosphamide only poorly (Sreerama and Sladek, 1993a, 1994a, b; Rekha *et al.*, 1994; Sladek *et al.*, 1995; Sreerama *et al.*, 1995a, b). When the ability of the enzyme to catalyse this reaction was normalized by the ability of the same enzyme to catalyse the oxidation of benzaldehyde to benzoic acid, the ALDH-3 isolated from normal parotid glands behaved as did ALDH-3s isolated from other human normal cells/fluids (stomach mucosa and saliva) whereas ALDH-3s isolated from Warthin tumours and mucoepidermoid carcinomas behaved as did those isolated from other human tumour cells (MCF-7 and colon C cells) (Table 7).

DISCUSSION

ALDH-3, glutathione S-transferases and DT-diaphorase are found in human saliva (Sreerama *et al.*, 1995b). The origin of these enzymes is not known with absolute certainty, but the findings reported here provide further support for the notion that it is the salivary glands.

Relative to those in normal parotid glands, levels of ALDH-3, glutathione S-transferase and DT-diaphorase were constantly elevated in Warthin tumours and mucoepidermoid carcinomas, but were never elevated in pleomorphic adenomas, nor in an indifferentiated carcinoma or an adenocystic carcinoma originating in the parotid. According to the taxonomic classification proposed by Dardick and Burford-Mason (1993), most human salivary gland tumours can be placed into one of four groups, the neoplastic counterparts of (1) luminal and basal/myoepithelial cells; stroma of basal lamina and glycosaminoglycans absent, (2) luminal and basal/myoepithelial cells; stroma of basal lamina and glycosaminoglycans present, (3) luminal cells, and (4) basal/myoepithalial cells. Parotid gland tumours in which high levels of ALDH-3, glutathione S-

Table 5. Cofactor preferences of ALDH-3s purified from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands

		K_m (μM)				
Substrate (mM)	Cofactor (mM)	Stomach[a] mucosa	Whole[a] saliva	Parotid glands	Warthin tumours	Mucoepidermoid carcinomas
Benzaldehyde (4)	NAD (0.01–1)	54	40	42	38	45
	NADP (0.1–1.4)	1000	1250	1150	1100	1200

[a]K_m values for stomach mucosa and salivary ALDH-3s are from previous publications (Sreerama and Sladek, 1993a; Sreerama *et al.*, 1995b); they are included here for comparative purposes.

Table 6. Esterase activity of ALDH-3s purified from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands[a]

Source	Esterase activity (miu/mg)
Stomach mucosa[b]	9800
Whole saliva[b]	8390
Parotid glands	8250
Warthin tumours	8000
Mucoepidermoid carcinomas	8780

[a]The rate at which purified ALDH-3s catalysed the hydrolysis of *p*-nitrophenyl acetate (500 μM) to *p*-nitrophenol was determined. Each value is the mean of three determinations.

[b]Values for stomach mucosa and salivary ALDH-3s are from previous publications (Sreerama and Sladek, 1993a; Sreerama *et al.*, 1995b); they are included here for comparative purposes.

Table 7. Catalysis of aldophosphamide and benzaldehyde oxidation by ALDH-3s purified from various normal and neoplastic tissues/fluids: relative rates[a]

Source of ALDH-3	(nmol aldophosphamide oxidized/min per mg) (1000) / (nmol benzaldehyde oxidized/min per mg)
Stomach mucosa[b]	0.29
Whole saliva[b]	0.32
Parotid glands	0.41
Warthin tumours	3.03
Mucoepidermoid carcinomas	3.54

[a]Aldehyde dehydrogenase activity was quantified; aldophosphamide (160 μM) or benzaldehyde (4 mM) was the substrate and NAD (1 mM) was the cofactor.

[b]Values for stomach mucosa and salivary ALDH-3s are from previous publications (Sreerama and Sladek, 1994b; Sreerama *et al.*, 1995b); they are included here for comparative purposes.

transferase and DT-diaphorase are found (Warthin tumours and mucoepidermoid carcinomas) are thus classified as group 1 tumours, and at least two of the parotid gland tumours in which ALDH-3, glutathione S-transferase and DT-diaphorase levels are low (pleomorphic adenomas and the adenocystic carcinoma) can be classified as group 2. Thus, it is tempting to speculate that the inability to synthesize and accumulate basal lamina and glycosaminoglycans, and increased levels of ALDH-3, glutathione S-transferases and DT-diaphorase, are interdependent, but how is not obvious.

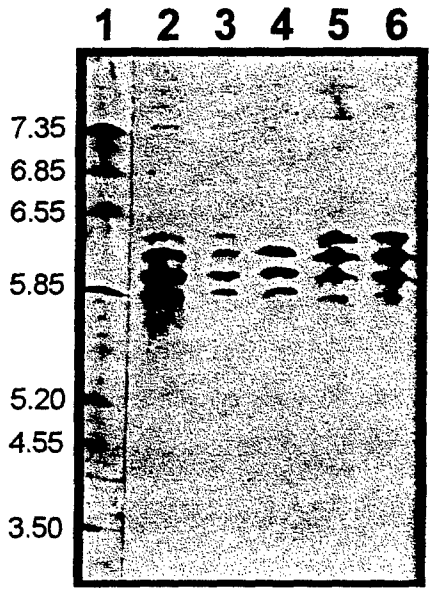

Fig. 4. Isoelectric focusing of ALDH-3s purified from normal parotid glands, and Warthin tumours and mucoepidermoid carcinomas of parotid glands. Purification and isoelectric focusing of ALDH-3s were as described in text. Subjected to isoelectric focusing were pI standards (lane 1), and amounts of the ALDH-3 purified from stomach mucosa (lane 2), normal parotid glands (lane 3), Warthin tumours of parotid glands (lane 4), mucoepidermoid carcinomas of parotid glands (lane 5) and saliva of a healthy adult human (lane 6), sufficient to generate 5–10 nmol NADH/min (as determined by spectrophotometric assay) when benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively. Lane 1 was stained for the presence of proteins with Coomassie Brilliant Blue R-250. Lanes 2–6 were stained for aldehyde dehydrogenase activity; benzaldehyde (4 mM) and NAD (4 mM) were used as the substrate and cofactor, respectively.

Mucoepidermoid carcinomas and Warthin tumours originate from parotid gland excretory and striated duct cells, respectively, whereas pleomorphic adenomas and adenocystic carcinomas originate from parotid gland intercalated duct/acinar cells (Batsakis, El-Naggar and Luna, 1992). Levels of glutathione S-transferase π are relatively high in excretory duct cells, somewhat lower in striated duct cells and still lower in intercalated duct cells; the enzyme was not detected in acinar cells (Zieper *et al.*, 1994). This suggests that mucoepidermoid carcinomas and Warthin tumours are derived from normal parotid gland cells in which ALDH-3, glutathione S-transferase and DT-diaphorase levels are relatively high, whereas pleomorphic adenomas and adenocystic carcinomas are derived from normal parotid gland cells in which, at best, levels of these enzymes are low.

Classification of salivary gland tumours as mucoepidermoid carcinomas on the basis of histopathological criteria is at present problematic (Burgess *et al.*, 1993); establishing that cellular levels of ALDH-3, glutathione S-transferases and/or DT-diaphorase are, or are not, elevated may be of assistance in that regard. Unknown is whether salivary levels of these enzymes mirror those of salivary gland tumours. If they invariably do, measurement of salivary levels of these enzymes could also be of, at least, preliminarily diagnostic value. However, a finding that salivary levels of these enzymes are elevated could be misleading, as such elevations are known to be effected by ingestion of various dietary constituents, e.g. coffee and broccoli (Sreerama *et al.*, 1995b). Also of diagnostic potential is our finding

that the ALDH-3 present in Warthin tumours and mucoepidermoid carcinomas, as well as that present in various other human tumours (Sreerama and Sladek, 1994b; Sladek *et al.*, 1995), appears to be a, albeit subtle, variant of that found in normal tissues/fluids, i.e., it appears to be tumour-specific. Release of such an enzyme by the salivary gland tumours into the saliva could allow early, non-invasive, detection of such tumours if a suitable assay, such as enzyme immunosorbent, could be developed.

In agreement with the reports of others (Corrigall and Kirsch, 1988; Campbell *et al.*, 1991; Zieper *et al.*, 1994), we did not detect the presence of glutathione S-transferase μ in all of the normal and neoplastic salivary gland samples that we examined. This is not surprising, as it has been shown that some 50% of the general population lacks the gene that codes for this enzyme (Board, 1981; Seidegard *et al.*, 1988; Comstock *et al.*, 1990; Harada *et al.*, 1992). Such individuals are thought to be at high risk for developing cancers of larynx, lung, stomach, colon and bladder (Seidegard *et al.*, 1990; Harada *et al.*, 1992; Lafuente *et al.*, 1993; Brockmoller *et al.*, 1994; Katoh *et al.*, 1995; Szarka *et al.*, 1995). Glutathione S-transferase μ is present in the saliva of some, but not all, individuals (Sreerama *et al.*, 1995b). It may well be that a total lack of glutathione S-transferase μ in the saliva reflects the deletion of the gene that codes for this enzyme in such individuals. That being the case, the presence or absence of glutathione S-transferase μ in the saliva could be determined, e.g. by enzyme immunoassay to ascertain non-invasively whether an individual lacks the relevant gene and is thus at high risk for developing certain cancers.

Standard treatment of salivary gland tumours is surgical removal followed by, in selected cases, radiotherapy. The 5-year survival of patients with high-grade salivary gland tumours such as adenocystic carcinomas, adenocarcinomas, mixed maligant tumours and mucoepidermoid carcinomas is only around 50%. In large part, this is because salivary gland tumours often metastasize, for example to the lungs, the most favoured site (Airoldi *et al.*, 1994, and references cited therein). Whereas locoregional recurrences can usually be successfully managed with additional surgery and/or radiotherapy, more distant metastatic tumours cannot, because their location be adequately pinpointed. In such cases, combination chemotherapy is used. Most often used for this purpose are cyclophosphamide, mitomycin C, cisplatin, methotrexate, adriamycin and 5-fluorouracil (Airoldi *et al.*, 1994, and references cited therein). Combinations of cyclophosphamide, cisplatin and other drugs are of therapeutic value in the treatment of most salivary gland tumours, but such combinations are of limited value in the treatment of mucoepidermoid carcinomas (Rentschler *et al.*, 1977; Creagan *et al.*, 1983; Dimery *et al.*, 1990; Airoldi *et al.*, 1994). Almost certainly contributing to the lack of sensitivity to cyclophosphamide and cisplatin on the part of mucoepidermoid carcinomas are the high levels in these tumours of ALDH-3 and glutathione S-transferases, as these enzymes catalyse the detoxification of cyclophosphamide and cisplatin, respectively (reviewed in Tsuchida and Sato, 1992; Sladek, 1993; Sreerama and Sladek, 1994a; Sreerama *et al.*, 1995b). That being the case, addition of agents to the chemotherapeutic protocol that inhibit these enzymes should serve to sensitize these tumour to cyclophosphamide and cisplatin.

Acknowledgements—This work was supported by USPHS Grant CA 21737, Bristol-Myers Squibb Company Grant 100-R220 and DOA DAMD17-94-J-4057. Descriptions of parts of this investigation have appeared in abstract form (Sreerama, L., Hedge, M. and Sladek, N. E. (1994) *Proc. Am. Assoc. Cancer Res.* **35**, 84).

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